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Blackleg of Canola in Alberta

Investigations on Biology, Epidemiology and Management

Prem D. Kharbanda, Ph.D., P.Ag.

Alberta
ENVIRONMENTAL CENTRE

BLACKLEG OF CANOLA IN ALBERTA:

Investigations on Biology, Epidemiology

and Management

by

P. D. Kharbanda, Ph.D., P.Ag.
Alberta Environmental Centre
Bag 4000, Vegreville, Alberta
T9C 1T4

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ABSTRACT

Blackleg, caused by the highly virulent strain of *Leptosphaeria maculans* (Desmaz.) Ces. & de Not. [conidial state= *Phoma lingam* (Tode: Fr.) Desmaz.], is a destructive disease of canola and has inflicted serious losses to *Brassica* crops around the world. In Canada, blackleg was first recorded in Saskatchewan in 1975. In Alberta, we first discovered the disease in one field near Vermilion in 1983. Our surveys conducted over the past several years have revealed that the disease has gradually spread throughout Agricultural Regions 4 and 3 in north-central Alberta.

The disease spreads with infected seeds and by the wind-borne ascospores and pycnidiospores of the fungus that develop on the overwintering infected stubble. Our survey results also indicate that the disease was introduced into Alberta most likely with infected seed and that ascospores did not play a major role in the disease spread in Alberta until 1989.

The fungus exhibits considerable variability in cultural characteristics; 350 isolates collected in Alberta could be grouped into five categories on the basis of overall cultural characteristics and rate of growth on potato dextrose agar. One representative isolate from each of the five categories was further tested for pathogenic variability and determination of race structure in *L. maculans*. None of the plant species belonging to the several genera of Crucifereae tested were found suitable to differentiate races of *L. maculans*. All commonly cultivated *Brassica* cultivars were found highly susceptible to blackleg, whereas most of the common Cruciferous weeds tested developed resistance to the fungus soon after emergence. *Erucastrum gallicum* (dog mustard) was found as a new host of *L. maculans*.

To provide information on blackleg tolerance of the recently licensed canola cultivars under Alberta conditions, research demonstration plots were set up at two farms naturally infested with blackleg in Alberta Agriculture Regions 3 and 4 in the summer of 1992. Under heavy blackleg disease pressure, *Brassica napus* cultivars Cyclone and Legend provided good yield and disease resistance, whereas Westar was totally destroyed by the disease. Compared with Westar, Tobin (*B. rapa*) developed significantly less blackleg. There was a significant negative correlation between blackleg disease severity and loss and in percent oil content of seeds; the loss could be estimated with the equation $Y = 0.1151 - 0.2511\ X$, where X represents the disease severity on a scale of 0 to 5.

To develop a fungicidal control program of the disease, several fungicides were evaluated in the laboratory, growth chamber and field as seed treatments and foliar sprays for their protective and curative action. Seed treatment with either of the fungicides, benomyl (Benlate® 50 WP), carbathiin + thiram (UBI-2390-2, 33.3 FL), iprodione (Rovral® ST 16.7 FL), prochloraz (Sportak® 20 SN), thiabendazole (Mertect® 45 FL), or tolclofos-methyl (Rizolex® 50 WP), significantly suppressed seed-borne *L. maculans* in agar plates. In the growth chamber, iprodione and prochloraz seed treatments effectively protected seedlings from infection up to 21 days after seeding. In field tests, however, none of the seed treatments prevented infection in seedlings artificially inoculated with the pathogen 15 days after seeding. Iprodione or prochloraz, sprayed once either four days before foliage inoculation or four days after stem inoculation with *L. maculans* in a growth chamber test, significantly controlled the disease. In field experiments conducted over two years (1988 and 1989), prochloraz (500 g a.i./ha) was the most effective fungicide under conditions of artificial inoculation. However, with natural infection and heavy disease pressure, two sprays of prochloraz at growth stages 2.5 and 4.1 failed to control the disease.

Field experiments were also conducted in 1990 and 1991 to develop control procedures for the disease by determining the timing of a fungicidal foliar spray using an estimate of the ascospore population in the air, and by utilizing partial resistance to blackleg present in the recently licensed canola cultivar Legend (*B. napus*). Foliar sprays consisted of the fungicides prochloraz and iprodione.

The population of ascospores in the air was determined by using a Burkard 7-day volumetric spore trap. Results of spore trapping indicated that i) ascospores were captured between May and September each year, ii) the population of ascospores did not increase as the growing season progressed and did not seem to peak at any particular time; however, the number of ascospores captured was considerably higher on the days immediately following precipitation of about 5 mm or more, and iii) numbers of ascospores captured were higher in 1991 than in 1990.

The disease severity of blackleg was higher in 1991 than in 1990. A significant control of the disease was obtained only in 1990 when prochloraz was sprayed before the crop was six weeks old. In 1991, spraying any time during the crop season did not control the disease. In the cultivar Legend, which developed significantly less disease than Westar, iprodione was

ineffective and two sprays of prochloraz were required to significantly reduce the disease; however, there was no significant increase in yield due to any fungicide treatment in either cultivar.

Biological control of the disease was attempted with *Penicillium verrucosum* recovered as an aerial contaminant. High performance liquid chromatographic (HPLC) analysis of the culture filtrate (6 weeks old) of the fungus *P. verrucosum* yielded an antifungal compound with a retention time of 10.6 minutes. Based on high-performance liquid chromatography, nuclear magnetic resonance, ultra-violet and mass spectral analysis, this compound was identified as citrinin. The metabolite was inhibitory to *L. maculans, Sclerotinia sclerotiorum*, and *Rhizoctonia solani* but not to *Alternaria brassicae* and *Fusarium equiseti*. Canola leaves from six-week-old plants, when inoculated with a mixture of conidia of *P. verrucosum* and *L. maculans*, developed blackleg which was much less severe than the disease which developed when inoculated with conidia of *L. maculans* alone. However, the reported nephrotoxic nature of citrinin makes it a non-candidate for biocontrol of blackleg and other diseases of canola.

1 INTRODUCTION

Blackleg caused by *Leptosphaeria maculans* (Desmaz.) Ces. & de Not. [conidial state *Phoma lingam* (Tode:Fr.) Desmaz.] has caused significant damage to *Brassica* crops in several countries within the last two decades. In 1966, severe blackleg on cabbage (*Brassica oleracea* L. var. *capitata* L.) in several eastern states in the U.S.A was traced to infected seed produced in Australia. In southern Australia, rapeseed was introduced as an alternative crop to wheat in 1968. Initial yields were promising, and the crop increased to about 200,000 ha in 1971. However, blackleg occurred widely in all the main growing areas in 1971, and caused a serious epidemic in 1972 (Bokor *et al.* 1975). Since then, the disease has become the major limiting factor in the establishment of the rapeseed industry in Victoria, New South Wales and Western Australia (Barbetti 1975a, Mcgee and Emmett 1977). From 1966 to 1968, prior to the development of resistant cultivars, blackleg was the major disease of rapeseed in parts of France, and caused a severe epidemic in 1966 in central France. Blackleg is one of the most important diseases of winter rapeseed in England and Germany. In 1976-77, stem canker was damaging in eastern and southeastern England (Gladders and Musa 1980).

In Canada, blackleg was confirmed first on oil rape [*Brassica napus* L., *B. campestris* (*rapa*) L.] in 1961, but it was considered of minor importance as strains that occurred were of relatively low virulence and only produced superficial stem lesions which did not cause any yield loss. However, in 1975, a virulent strain was isolated from rape stubble from two fields in east-central Saskatchewan. Its prevalence and incidence increased rapidly during the following years and severe localized stem canker outbreaks occurred in parts of the province in 1982 and 1984, causing yield loses from 10 to 25% (Petrie 1978, 1985). The disease was reported from 92% of Saskatchewan fields surveyed in 1989 (Jesperson 1989, 1990). The disease is quite widespread in Manitoba where about 61% of the fields surveyed were found infested in 1989.

In Alberta, the virulent strain of *L. maculans* was found for the first time in 1983 in a field near Vermilion (Kharbanda and Stevens 1983). The disease has since spread throughout the northeastern region of the province.

The canola cultivars presently grown are all susceptible to blackleg but sources of resistance have been identified and are currently being used in canola breeding programs. A considerable amount of work has been done on occurrence, epidemiology and control of this disease at the Alberta Environmental Centre, Vegreville. The purpose of this monograph is to

compile this information with a view to developing an integrated disease control strategy to prevent and minimize crop losses.

2 SYMPTOMS OF BLACKLEG OF CANOLA

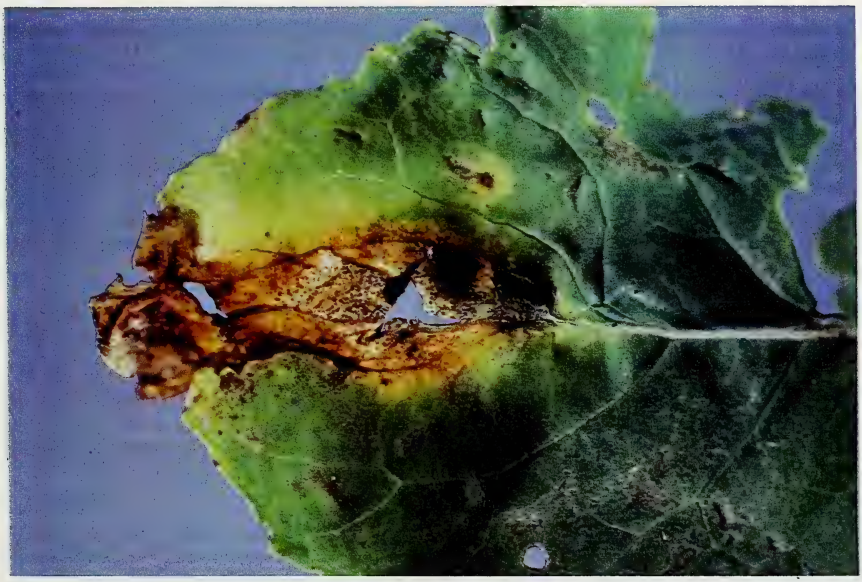
Leptosphaeria maculans attacks the leaves, stems and seed pods of several *Brassica* spp., and causes a variety of symptoms. Seedlings attacked early in the season develop seedling blight (Plate 1a). On turnip and Swede bulbs, and on cabbage stems, it produces black dry rot in field or storage.

On canola, leaf spots appear as dirty white, round to irregular lesions speckled with numerous black pycnidia which in the presence of abundant moisture, exude a pink-amethyst ooze containing spore masses (Plate 1b).

On the stem, grey to tan-coloured lesions, usually with a black margin, develop near the base of the scar remaining from fallen infected leaves. These lesions develop into crown canker causing extensive tissue damage, and may expand in size until the plant is severed at ground level and lodges. Less severely affected plants may remain standing but ripen prematurely; pods fail to fill and develop shrivelled seeds. Pycnidia are frequently produced on the cankered area and can also ooze pink spore masses (Plate 2a). Occasionally, some infected stems may not show any external symptoms but internally there is marked blackening of the tissue (Plate 2b). Infection of pods (Plate 2c) in field is not very frequent, but when it happens, it causes premature pod splitting, resulting from unequal drying out of infected and uninfected portions of the halves. Greyish mycelium may be present inside the pod. Seeds beneath the lesions are shrunken, unsound and pale grey. Pycnidia of the fungus are sometimes visible to the naked eye on the seed surface (Plate 3a).

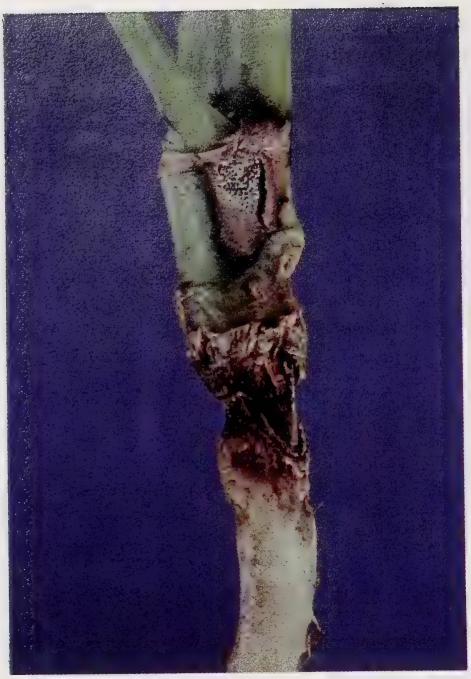
A weakly virulent strain of the fungus infects plants near maturity, causing lesions which tend to be shallow, do not have a distinct black margin and contain relatively fewer, scattered pycnidia (Plate 3b). The weakly virulent strain causes only limited tissue damage. On the other hand, the more aggressive (or highly virulent) strain produces deep lesions resulting in extensive tissue damage. The lesions have a distinct black margin, and contain numerous pycnidia which may be grouped together.

a. Young plants.



b. Leaf.

Plate 1. Blackleg of canola symptoms (A).



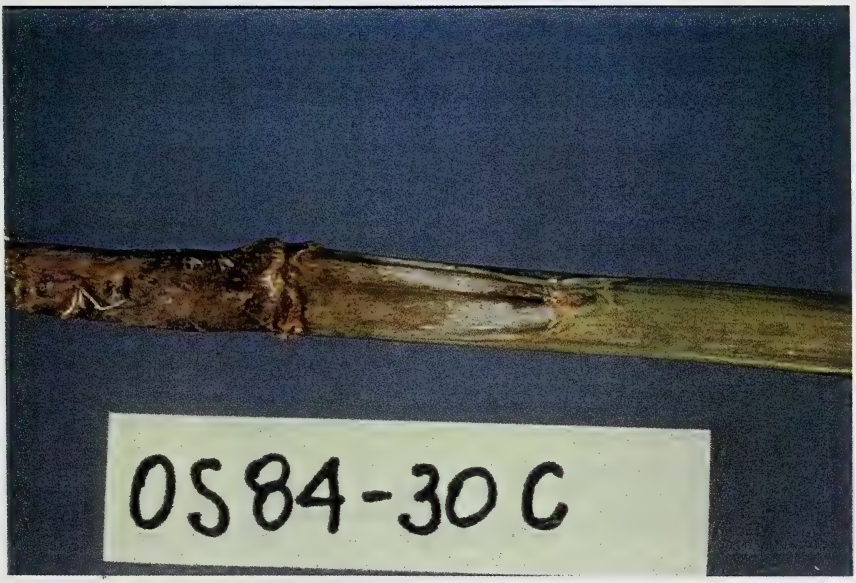
a. Stem

b. Stem (internal symptoms).

c. Pod.

Plate 2. Blackleg of canola symptoms (B).

a. Seeds.



b. Stem infected with weakly virulent *Leptosphaeria maculans*.

Plate 3. Blackleg of canola symptoms (C).

3 THE PATHOGEN

The taxonomic classification of *L. maculans* could be summarized as follows (Ainsworth *et al.* 1973):

Division: *Eumycota* (Filamentous fungi)

Sub-Division: *Ascomycotina* (Sexual reproduction resulting in the formation of ascospores borne in asci)

Class: *Loculoascomycetes* (Asci bitunicate; ascocarp an ascostroma developing into pseudothecium)

Order: *Pleosporales* (Locules generally polyascal; pseudoparaphyses present)

Family: *Pleosporaceae* (Pseudothecia mid-sized to large containing cylindrical asci among persistent pseudothecia, superficial erumpment, or, in or on stroma, ascospores one to many celled)

Genus: *Leptosphaeria* (Pseudothecial wall composed of a scleroplectenchyma of isodiametric, thick-walled cells, pseudothecial beak short, ascospores three to many septate brownish yellow to hyaline; mostly parasitic on stems of herbaceous plants)

The sexual stage of *P. lingam* was first discovered by Smith and Sutton (1964) as *L. maculans*. The fungus is heterothallic (Venn 1979) and forms on dead leaves, stems, and roots of crucifers. In culture, production of pseudothecia requires growing of opposite mating types under specialized conditions of temperature, near ultra violet light, and organic substrate (Mengistu *et al.* 1990).

The ascocarps (pseudothecia) on stems and leaves are immersed, becoming erumpent, globose, black, with protruding ostioles, 300-500µ diameter. Asci are cylindrical to clavate, sessile or short stipitate, eight spored, 80-125x15-22µ; ascus wall bitunicated. Ascospores are 5-septate, biseriate, cylindrical to ellipsoidal, ends mostly rounded, yellow brown, slightly or not constricted at the central septum, guttulate, 33-70x5-8µ. Pseudoparaphysis filiform, hyaline and septate (Punithalingam and Holliday 1972).

Phoma lingam, the asexual state belongs to the class Deuteromycetes or Imperfect Fungi, order Sphaeropsidales (conidia in pycnidia), and family Sphaeropsidaceae.

Pycnidia on stems and leaves are immersed, becoming erumpment, gregarious, variable in shape, convex, soon becoming depressed and concave, sometimes without any definite shape,

with narrow ostioles, 200-500μ across, wall composed of several layers of cells, thick walled on the outermost layer. Conidia hyaline, shortly cylindrical, mostly straight, some curved, guttulated, with one guttule at each end of the conidium, unicellular 3-5x1.5-2μ (Punithalingam and Holliday 1972).

3.1 Cultural and Pathogenic Variability

Leptosphaeria maculans exhibits considerable variability in virulence and in cultural characteristics. Henderson (1918) noted variability in cultural characteristics of three *L. maculans* isolates from Ohio and Wisconsin, U.S.A, cultured on several media and concluded that the strains belonged to the same species. Cunningham (1927) examined pathogenicity and cultural characteristics of about 400 isolates of *P. lingam* and classified them into two strains: (i) relatively slow growing and strongly pathogenic on *Brassica*, and (ii) faster growing and weakly pathogenic on *Brassica*. Pound (1947) also described a strain of *P. lingam* from Puget Sound area of western Washington (USA) that grew rapidly in culture, was a weak pathogen on cabbage plants, and produced a water-soluble yellow to brown pigment in culture. A number of other investigators since reported similar phenomena and identified two strains, weakly virulent (non-aggressive) and highly virulent (aggressive) (Bonman *et al.* 1981; Humpherson-Jones 1983; Koch *et al.* 1989; McGee and Petrie 1978).

Recognition of strains varying in virulence is the backbone in the development of crop cultivars with lasting resistance to that fungus in a disease management program. Not much information on existence of distinct races of *L. maculans* is available because differential hosts are not known. Koch *et al.* (1989) found that three *B. napus* cultivars, Westar, Quinta and Glacier, could be used as differential hosts. Using these differential hosts, Mengistu *et al.* (1991) could group about 100 isolates into four pathogenicity groups (PG), PG1, PG2, PG3 and PG4; however, specific races of *L. maculans* could not be defined.

To explore the compositions of *L. maculans* populations in Alberta, I evaluated cultural and pathogenic variability among *L. maculans* isolates collected between 1983-1988.

3.1.1 Cultural Variability

Isolates of *L. maculans* were obtained from infected plant stubble collected during annual surveys in north-eastern Alberta conducted since 1983. Infected plant tissue was surface sterilized in 1% NaOCl for 1 minute, and plated on V8-juice agar containing rose bengal and streptomycin sulfate (McGee and Petrie 1978). The resulting *L. maculans* colonies were sub-cultured on potato dextrose agar (PDA), single spored and preserved in PDA culture tubes at 4°C until further use.

Over 350 isolates collected between 1983-1986 were single spored. The isolates varied considerably in their morphological characteristics, amount of aerial mycelium, production of pycnidia, color of pigmentation in the medium, and overall colony appearance. The isolates were classified into 15 groups based on overall colony appearance and diameter (4 weeks growth). One representative isolate was chosen for further study.

3.1.1.1 Determination of Cultural Characteristics

Colony characteristics and rate of growth were observed on Difco PDA. A mycelial agar disc (4 mm) from the margin of a 10-day-old colony was placed in the centre of a 90 mm plate containing 20 mL of medium and incubated at 21° C. There were four replications for each isolate arranged in a completely randomized design. Two diameters at right angles were measured for each colony at 3-day intervals. The amount of aerial mycelium, color, concentration, and size of pycnidia, and pigmentation in the medium were noted after 21 days. The experiment was repeated once. For sporulation assessment, 90 mm plates containing 20 mL PDA were flooded with a 3 mL suspension containing $1x10^7$ conidia/mL and incubated for 10 days at 21° C. There were four replications for each isolate. Concentrations of harvested spores (final volume 100 mL) were determined by counting four samples from each replication with a hemacytometer. The experiment was repeated once.

Cultural characteristics of the 15 single-spored isolates tested are presented in Table 1. Rate of growth (over 21 days) was categorized from very slow to fast using the following scale:

Very slow: colony diameter less than 40 mm

Slow: colony diameter between 41 mm and 60 mm

Moderate: colony diameter between 61mm and 80 mm

Fast: colony diameter over 81 mm

All weakly virulent (WV) isolates were fast growing. Several virulent isolates varied considerably in terms of pycnidial production, aerial mycelium, colony morphology, rate of growth and production of pigment in the culture medium. Considering all these characteristics, the virulent isolates could be classified into five categories (Plate 4, Figure 1):

1. Very slow growing; fluffy, white mycelium; pycnidial production little (Isolate S)
2. Moderately fast growing; compact, white mycelium; very few pycnidia (Isolate N)
3. Slow growing; flat, grey mycelium; pycnidial production abundant (Isolates A,B,C,M)
4. Moderately fast growing; flat, white or grey mycelium; pycnidial production mostly abundant (Isolates F,G,H,J,K,L)
5. Moderately fast growing; compact, white mycelium; pycnidial production abundant (Isolates P,D)

Attempts were also made to study cultural characteristics and to group the isolates collected in 1987 and later years. All isolates exhibited enormous cultural variability; however, these could be placed in one of the five categories stated above. Due to the similarities of these isolates with those found in the previous years described above, their data are not presented in this report.

3.1.2 Pathogenic Variability

One representative isolate from each of the five categories (based upon morphological characteristics) was tested for virulence on *B. napus* cv. Westar and *B. rapa* cv. Tobin. A modified method of Helms and Cruickshank (1979) was followed:

Seeds of the cultivar to be tested were placed on the moistened surface of the soil mix in a 15 cm pot and covered with 150 mL perlite mixed with 50 mL conidial suspension (5×10^6 conidia/mL). The seeded pots were covered individually with plastic bags to maintain high humidity for 4 days. All treatments were replicated four times and arranged in a completely randomized design in a growth chamber. The number of infected seedlings (with typical pycnidia) in each pot were recorded at various time intervals until 28 days after seeding.

In both cultivars, the percentage of plants infected averaged between 60-80% for each isolate indicating that both of these cultivars are equally susceptible to the *L. maculans* isolates used under growth chamber conditions, and that these cultivars were not of much use as differential hosts to identify different races of *L. maculans*. Therefore, attempts were made to

Table 1. Cultural characteristics of 13 *Leptosphaeria maculans* isolates recovered in Alberta and an isolate from Saskatchewan on potato dextrose agar after 21 days incubation at 21°C.

Isolate Code	Isolate (original)	Pycnidial Production	Rate of Growth	Colony Diameter (mm)*	Aerial Mycelium	Pigment in Media
BL-S	Sask 86-1 V	little	very slow	33 I	dense, piled, cottony, grey, brownish exudate	brown
BL-A	BL-5 V	abundant	slow	60 F,G	flat, grey, raised at edges	dark brown
BL-B	BL-10 V	abundant	slow	57 G,H	flat, grey, raised at edges	brown
BL-C	BL-6 V	abundant	slow	59 G	flat, grey, raised at edges	brown
BL-D	BL-86-24 V	abundant	moderate	64 D,E,F,G	moderate aerial mycelium, white	light grey
BL-F	BL-9 V	few	moderate	63 E.F.G	flat, white	tan
BL-G	BL-86-31 V	abundant	moderate	69 B,C,D,E	flat, grey	light tan
BL-H	BL-86-28 V	abundant	moderate	73 B,C	whitish, flat	light tan
BL-J	BL-86-18 V	abundant	moderate	74 B	whitish, flat	tan
BL-K	BL-86-20 V	abundant	moderate	68 C,D,E,F	moderate aerial mycelium, white	light grey
BL-L	BL-86-27 V	abundant	moderate	71 B,C,D	whitish, flat	light tan
BL-M	BL-86-8 V	abundant	slow	53 H	grey,moderate aerial mycelium	light tan
BL-N	BL-15 V	very few	moderate	64 E,F,G	considerable aerial mycelium, white, compact	tan
BL-P	OS-86-19 V	abundant	moderate	70 B,C,D,E	moderate aerial mycelium, white	light tan
WV	BL-86-17 WV	abundant	fast	85 A	fat, dark grey	brown

* Means of four replications; means followed by the same letter are not significantly different according to Duncan's Multiple Range Test (P = 0.05).

Plate 4. Colony characteristics of five virulent *Leptosphaeria maculans* isolates and one weakly virulent isolate referred to in the text as: BL-S (1), BL-N (2), BL-B (3), BL-F(4), BL-P (5), and weakly virulent (x).

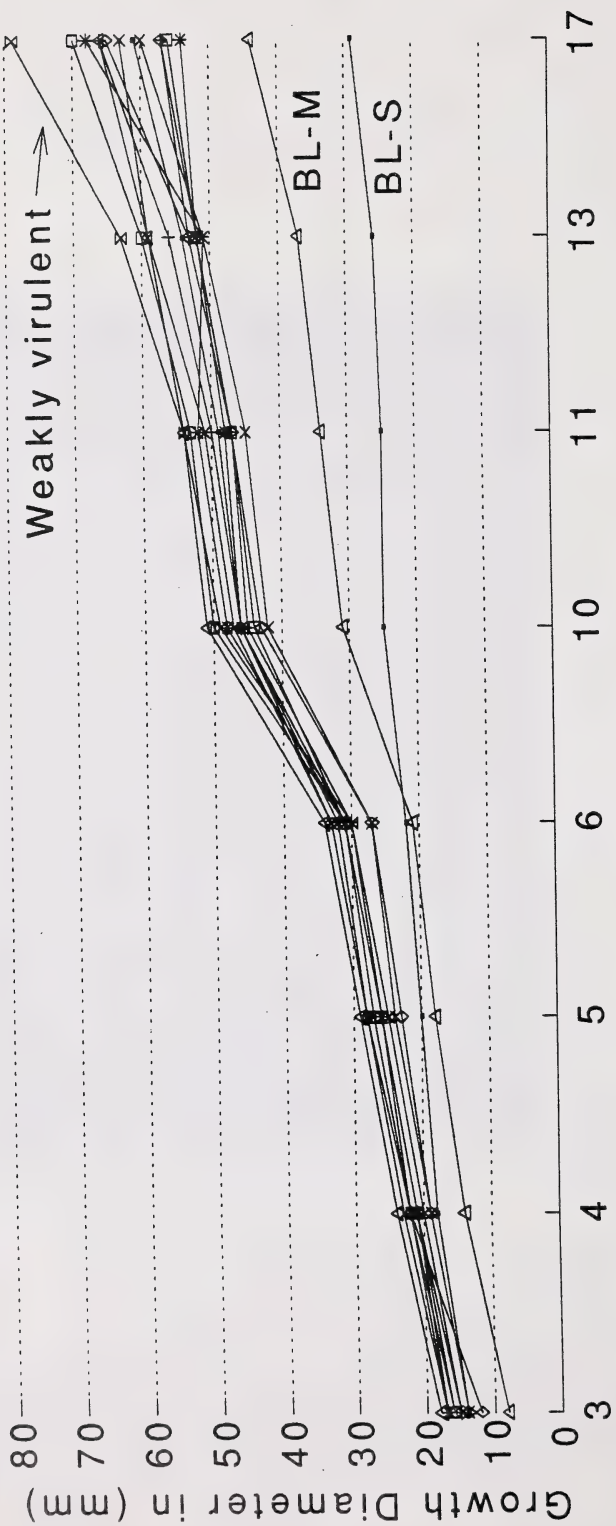


Figure 1. Growth rate of *Leptosphaeria maculans* isolates recovered in Alberta at 21°C on potato dextrose agar.

test virulence of the five morphologically distinct *L. maculans* isolates on different genera of Cruciferae including cruciferous weeds commonly prevalent in Alberta as well as several cruciferous vegetable crops; these plant species were chosen to obtain information on their susceptibility to blackleg and their role in the disease epidemiology.

3.1.2.1 Susceptibility of Cruciferous Weeds and Cultivated Vegetable Crops

The weeds and vegetables tested were: *Neslia paniculata* (ball mustard), *Lepidium densiflorum* (common peppergrass), *Erucastrum gallicum* (dog mustard), *Descurainia sophia* (flixweed), *Capsella bursa-pastoris* (shepherd's-purse), *Thlaspi arvense* (stinkweed), *Brassica kaber* var. *pinnatifida* (wild mustard), *Raphanus raphanistrum* (wild radish), *Erysimum cheiranthoides* (wormseed mustard), *Brassica oleracea* var. *botrytis* (broccoli), *Brassica oleracea* var. *capitata* (cabbage), *Brassica oleracea* var. *botrytis* (cauliflower), *Brassica napus* var. *napobrassica* (rutabaga).

To manage the growth chamber space and time used for inoculations, the experiment was conducted in several batches each including only two of the weeds and all the *L. maculans* isolates. Each batch also included *B. napus* cv. Westar as a susceptible check. To avoid problems associated with poor germination of weed seeds, the seeds were germinated in petri-plates and only viable seedlings were placed on the soil-mix at 10 seedlings per pot. Inoculations were done using two different techniques: (i) the germination inoculation technique described earlier (modified from Helms and Cruickshank 1979), and (ii) a foliar inoculation technique of Wood and Barbetti (1977) in which germinated seeds of cruciferous weeds, or seeds of other cruciferous species, were planted at 10 per pot in 15-cm-diameter fibre pots. At the two to three leaf growth stage (GS 2.3) (Harper and Berkenkamp 1975), seedlings were sprayed with a pycnidial suspension of *L. maculans* in 0.5% gelatin containing 5 x 10^6 pycnidia/mL. The pots were covered individually with plastic bags for 48 hours. The seedlings were examined for blackleg infection 7 days after inoculation; the final data were taken 21 days after inoculation.

Results are summarized in Tables 2 - 5. In the foliar inoculation tests, all the weeds tested showed resistance to most of the *L. maculans* isolates, except wild mustard which showed low susceptibility to isolates BL-N and BL-P, and stinkweed which showed low susceptibility

to BL-A, BL-N and the Saskatchewan isolate BL-S (Table 2). Wild mustard was previously reported to be a host of *L. maculans* (Petrie and Vanterpool 1968).

In the soil inoculation tests which provided heavy disease pressure on the seedlings, cotyledons and root of several weeds developed infection (Table 3; Plate 5, 6). Dog mustard was the most susceptible of all the weeds tested; the cotyledons on some plants were severely infected by all the isolates except BL-S (Plate 5a). This is the first report on susceptibility of the genus *Erucastrum* to *L. maculans*. Cotyledons of wild radish were also susceptible to several isolates (Plate 5b). The significance of these weeds in the epidemiology of blackleg is uncertain, however, because the weeds seem to have developed resistance to blackleg soon after emergence as evident from the foliar inoculation test in which leaves did not develop any infection.

Variable expression of disease symptoms by the weeds to the single-spored isolates tested shows genetic variability among different *L. maculans* isolates as well as variability in the genetic make-up of the individual weed populations. Genetically homogenous populations of some of the weeds such as dog mustard, flixweed, stinkweed, and wild radish may be useful to distinguish *L. maculans* races.

All the cultivated cultivars (vegetables) belonging to several *Brassica* spp., except rutabaga, were found to be extremely susceptible to the three *L. maculans* isolates that were tested by either soil or foliar inoculation techniques (Table 4); rutabaga developed infection only when planted in inoculated soil. These vegetable crops could play an important role in blackleg epidemiology because blackleg-infected seed could spread the disease to new uninfested locations hundreds of miles away. In a region intensively cultivated with canola, such as the Peace Region of Alberta, this source of infection could be extremely important in introducing the disease.

Due to the extreme susceptibility of the cultivated genera of the *Brassica* spp., their use as differential hosts to determine races of *L. maculans* is only limited. To further investigate if more than one race of virulent strain of *L. maculans* exists in Alberta, the pathogenicity of the six selected isolates was tested on genetically homogenous populations of *B. napus* cultivars Westar, Quinta, and Glacier. These cultivars were recently used by Mengistu *et al.* (1991) to differentiate pathogenicity groups (PG), PG1, PG2, PG3, PG4. Reaction of these pathogenistic groups as described by Mengistu *et al.* (1991) is summarized below:

	Westar	Quinta	Glacier
PG1	--	--	--
PG2	+	--	--
PG3	+	+	--
PG4	+	+	+

In our tests, a representative isolate of each group, obtained from the University of Wisconsin, was included to serve as a check. A cotyledon inoculation technique described by Mengistu *et al.* (1991) was followed. The disease severity was rated from low to severe based on the lesion size on cotyledons (Plate 7). The results presented in Table 5 show that the representative isolates belonging to different PGs did not give the reaction as reported (Mengistu *et al.* 1991). Therefore, these cultivars used in our studies did not prove to be truly differential hosts. The results indicate that further work is required to locate differential hosts to identify *L. maculans* races.

In further attempts to identify *L. maculans* races, their differential susceptibility to various fungicidal groups was explored. Fungicides tested were: Benlate (benomyl), HWG 1608 (ethyltrianol), Rovral (iprodione), San 619 (cyproconazole), Sportak (prochloraz), and Tilt (propiconazole). The requisite amount of a fungicide was dissolved in 1 mL of acetone and incorporated into 19 mL of PDA (50° C) in an 90 mm petri-plates so as to obtain a final concentration of 50 mg/mL. On the solidified agar, a 4 mm mycelial plug of actively growing *L. maculans* culture was placed in the centre of each plate. Colony diameters were measured 21 days after incubation at 21° C in the dark.

Results are presented in Table 6. On the basis of growth attained on Sportak and Rovral, the *L. maculans* isolates used could be classified into four genetically diverse groups: (i) WV, (ii) BL-A, (iii) BL-N, and (iv) BL-B, BL-F, and BL-S. Further work is required on using such fungicidal markers to identify genetically diverse races of *L. maculans*.

a. Dog mustard (*Erucastrum gallicum*).

b. Wild radish (*Raphanus raphanistrum*).

Plate 5. Symptoms of blackleg on cruciferous weeds artificially inoculated with *Leptosphaeria maculans* (BL-A).

a. Wild mustard root (*Brassica kaber*) in infested soil.

b. Stinkweed (*Thlaspi arvense*) reaction to foliar inoculation.

Plate 6. Symptoms of blackleg on cruciferous weeds artificially inoculated with *Leptosphaeria maculans* (BL-A).

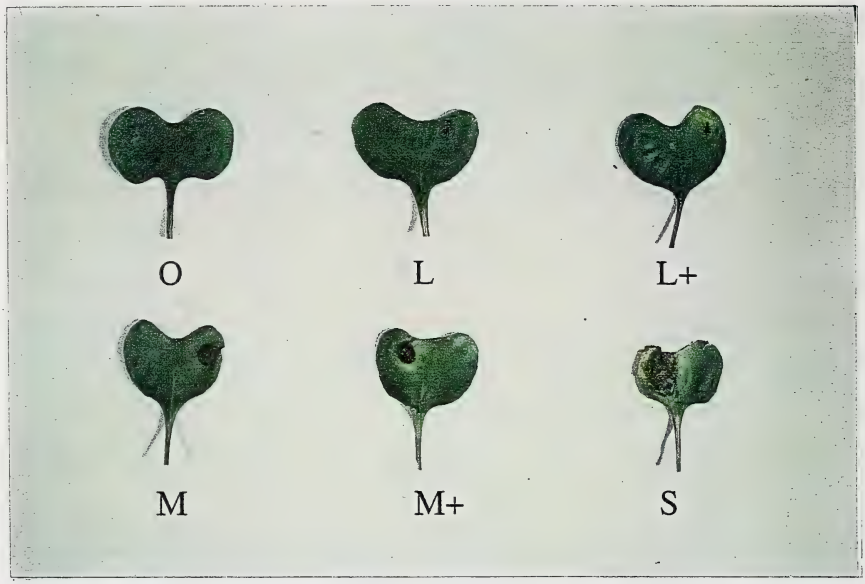


Plate 7. Ten day old cotyledons of *Brassica napus* cultivars showing differential susceptibility to different isolates of *Leptosphaeria maculans*: 0(R) = resistant; L = low; M = moderate; S = severe.

Table 2. Reaction of cruciferous weeds to infection by *Leptosphaeria maculans* strains: Foliar Inoculation *

SPECIES	BL-A	BL-B	BL-F	BL-N	BL-P	BL-S
Wild mustard	R	R	R	L	L	R
Shepherd's-purse	R			R		R
Flixweed	R		R	R	R	R
Common peppergrass	R			R		R
Dog mustard	R	R	R	R	R	R
Ball mustard	R	R	R	R	R	R
Wormseed mustard	R					R
Stinkweed	L	R	R	L	R	L
Wild radish	R	R	R	R	R	R

* Disease rating: R = 0% plants infected (PI); L = 1-30%.

Table 3. Reaction of cruciferous weeds to infection by *Leptosphaeria maculans* strains: Soil Inoculation*

SPECIES	BL-A	BL-B	BL-F	BL-N	BL-P	BL-S
Wild mustard	L	L	L	L	R	L
Shepherd's-purse	R			R		R
Flixweed	L	R	L	R	L	R
Common peppergrass	R			R		R
Dog mustard	S	S	S	M	S	L
Ball mustard	R	R	R	R	R	R
Wormseed mustard	R					R
Stinkweed	L	L	R	R	R	R
Wild radish	M	M	R		S	L

* Disease rating: R = 0% plants infected (PI); L = 1-30% PI; M = 31-70% PI; S = 71-100% PI.

Table 4. Reaction of vegetable crops to infection by three *Leptosphaeria maculans* strains applied with two inoculation techniques *

Soil Inoculation			
SPECIES	BL-A	BL-F	BL-P
Broccoli	S	S	S
Cabbage	S	S	S
Cauliflower	S	S	S
Rutabaga	S	S	S
Foliar Inoculation			
SPECIES	BL-A	BL-F	BL-P
Broccoli	S	S	S
Cabbage	S	S	S
Cauliflower	S	M	S
Rutabaga	R	R	R

* Disease rating: R = 0% plants infected (PI); L = 1-30% PI; M = 31-70% PI; S = 71-100% PI.

Table 5. Reaction of canola cultivars (*Brassica napus*) to infection by *Leptosphaeria maculans* isolates belonging to known and unknown pathogenicity groups (PG) *

ISOLATE	Westar	Glacier	Quinta
PG-4	S - M	S	S
PG-3	S - M	S - M	M
PG-2	S	S	S
BL-A	S	M	S
BL-B	L	L	L
BL-F	S	S	L
BL-N	L	L	M
BL-P	L	M	S
BL-S	S	L - M	L

* Disease rating: L = low; M = medium; S = severe (See Plate 7).

Table 6. Growth of different strains of *Leptosphaeria maculans* on potato dextrose agar plates amended with one of several fungicides tested.

	Fungicides Tested*						
ISOLATE	I	II	III	IV	V	VI	VII
	Colony mean diameter (mm)						
WV	13.5	8.75	5.5	4.25	11.75	26	67.5
BL-A	4	4	4	4	4	9	44.5
BL-B	4	5.5	4	5	5	19.75	40.5
BL-F	4	5	5.5	4.5	5.25	14.75	45.25
BL-N	0	0	4.75	0	0	0	30.75
BL-P	5	7	6	6.5	7.75	24.25	45.75
BL-S	4.5	6.75	5.25	4.25	5.5	29.75	40

I = cyproconazole; II = propiconazole; III = prochloraz; IV = benomyl; V = ethyltrianol; VI = iprodione; VII = Check (no fungicide).

4 DISEASE EPIDEMIOLOGY

The fungus overwinters on crop stubble and within and on infected seed. All infected seeds do not give rise to infected seedlings and usually the infection is very low, about 0.6%. However, depending upon host susceptibility and suitable environmental conditions, even 0.6% seedling infections could result in serious disease levels. In areas where the virulent strain is established, seed infection is of little importance to disease development but it could be the means of introducing the pathogen in infested regions.

On the first year crop stubble, *L. maculans* overwinters as mycelium and pycnidiospores and forms perfect stage pseudothecia some time before the following spring. It is not known what conditions trigger the formation of pseudothecia. The fungus is heterothallic and, therefore, (+) and (-) strains of the fungus need to cross for successful formation of pseudothecia (Venn 1979). Ascospores are ejected and are wind-borne over long distances. These wind-borne ascospores are the major source of infection and their release occur at intervals following rainy periods (Barbetti 1975b, Gladders and Musa 1980).

Ascospores and pycnidiospores primarily infect young rapeseed plants, resulting in cotyledon, leaf and stem infection. The earlier the infection takes place, the more severe the disease in adult plants; canola plants are most susceptible to infection from seedlings to the six-leaf stage of growth (Barbetti 1975b, McGee and Petrie 1979).

In crops sown on or adjacent to fields in which rapeseed was grown in the previous year, the disease sometimes becomes severe enough to cause crop failure (Gladders and Musa 1980, McGee and Emmett 1977). Even a very small amount of infected stubble may be sufficient to initiate an epidemic by ascospores. Pycnidiospores produced subsequently in these primary lesions can be carried in a moisture film or by rain splash from leaf spots to the leaf axil or by spread of mycelium down the petiole to the stem (Hammond *et al.* 1985). Although the pycnidiospores do not become airborne, they can be responsible for leaf, stem and crown infections, where they can then play a major role in the spread and establishment of the disease within a rape crop (Barbetti 1976, McGee 1977). High rainfall in conjunction with long periods of continuously wet days favour the spread of disease. The lack of sporulation at low temperatures may reduce the rate of spread of the pathogen in the field, and so slow down secondary disease spread within a crop (Barbetti 1976).

The pathogen can survive on crop residue for about five years and it has been shown that ascospores can be discharged for at least three years after the original crop was grown. Under normal field conditions, however, only a small proportion of crop residue survives more than one year after harvest (Barbetti 1975b, McGee 1977).

5 OCCURRENCE AND SPREAD OF BLACKLEG IN ALBERTA

Field surveys to monitor blackleg of canola were initiated by the Alberta Agriculture after virulent strains of *L. maculans* was found in Saskatchewan in 1976. Alberta Environmental Centre assumed the responsibility for the disease surveys in the north eastern region (Alberta Agriculture, Region 4) in 1981. Since the disease had spread in Saskatchewan up to about 20 km east of Lloydminster in 1981, probability of it continuing to spread westward was certain. As well, large quantities of seed were being imported into Alberta by farmers in the Lloydminster area. The purpose of the surveys was to determine the presence of the virulent strain of *L. maculans*. (Evans *et al.* 1990, 1991, 1992, 1993; Kharbanda and Stevens, 1983 and Kharbanda *et al.* 1988 and 1989.)

5.1 Survey: Methodology

Unless mentioned otherwise, canola fields were surveyed soon after swathing during the first two weeks in August of each year. One field was chosen at random about every 15 km along the secondary highways in north-central Alberta. Each field was sampled by traversing along the path of an inverted V and examining canola plants at five spots about 30 m apart. At each spot, 10 plants were examined visually, and the plants suspected to be infected with blackleg were collected for laboratory testing. The virulent nature of blackleg was confirmed by cultural methods (McGee and Petri 1978). Blackleg severity was assessed from low to severe based upon the depth and size of stem lesions: healthy = no lesions; low = small basal lesions; moderate = lesion up to several cm long; severe = stem girdled but not severed at base; very severe = stem severed, plant lodged. From 1989 onward, most of the surveys were carried out by Agricultural Fieldmen who forwarded all samples suspected of blackleg infection to the nearest Alberta Agriculture Regional Crop Laboratories in Brooks, Fairview or Olds, or at Vegreville (Alberta Environmental Centre); however, final diagnosis was made at Vegreville.

5.2 Survey: Results

The spread of blackleg of canola in Alberta is depicted in Figures 2-5. In 1983, blackleg was found for the first time near Vermilion, about 60 km west of the Saskatchewan border at Lloydminster, Alberta (Kharbanda and Stevens 1983). The disease was found in six fields (11% of fields surveyed) in 1984, and in twelve fields (33%) in 1985, around Paradise Valley about 60 km south-west of Lloydminster. In 1986, the disease was found in 50% of the fields surveyed in the same general area as in the previous years. In 1987, environmental conditions were quite dry and the disease was found in 30% of the fields surveyed; however, it had dispersed to a wider area in the Region (Figure 2).

In 1988, blackleg was discovered in 90% of the fields surveyed and it covered the area from Bonnyville and Provost in the east and to Smoky Lake and Camrose in the west. The disease was particularly severe around Paradise Valley, Viking and Sedgewick. One of the farms lost over 60% yield. This was the first year the significant economic losses due to blackleg was observed on a farm scale in Alberta. In 1989, in contrast to previous years results, the disease was found only in 54% of the fields, even though the environmental conditions for blackleg development were very conducive due to frequent rains. The most probable reason for this reduction in number of infested fields appears to be that in response to the intensive blackleg awareness program launched by Alberta Agriculture a majority of the farmers in this region did not grow canola on fields where canola was cropped in the past 2-3 years and had adopted other cultural control measures for the disease.

Our 1990 and 1992 survey results show that the disease has gradually spread in most of Region 4 and Region 3; however, the zone of greatest disease incidence has shifted from Paradise Valley to Sedgewick where the disease has been located mostly on farms where canola was planted more frequently than once in three years (I.R. Evans, personal communication).

To determine the primary source of blackleg infection in Alberta, pertinent information was collected on infected fields in 1984, 1985, and 1986. Results are presented in Tables 7, 8, 9. Since the seed planted was treated, and infested fields were very close to the Saskatchewan border, it was presumed that the primary source of infection was ascospores that had blown westward. However, because wind directions mostly prevail from the north-west in this region, it was suspected that perhaps infected seed had also played some role in the disease spread. In 1985, therefore, several seed samples from co-operating farmers were tested at the Environmental

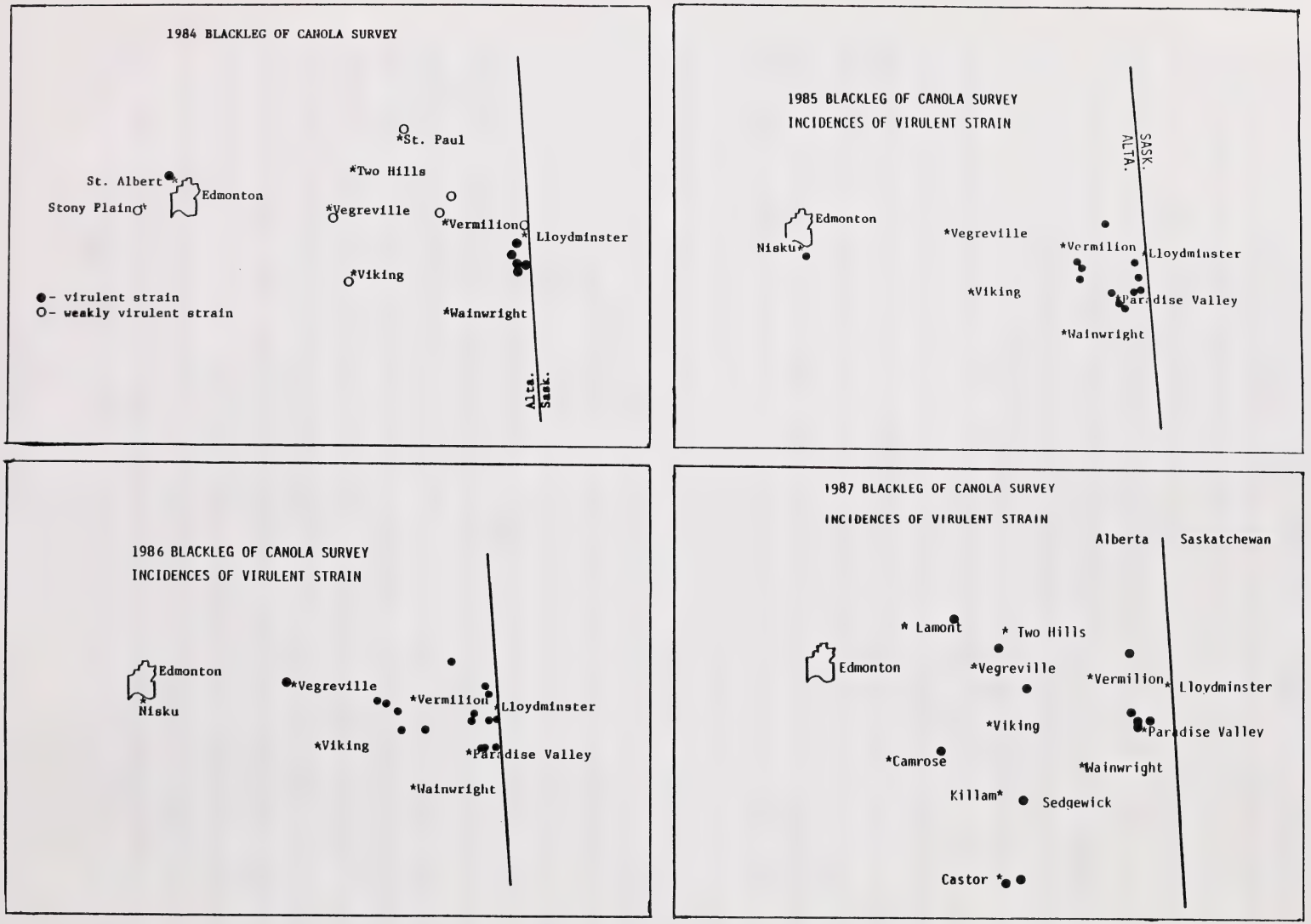


Figure 2. Blackleg of canola distribution in Alberta, 1984-87.

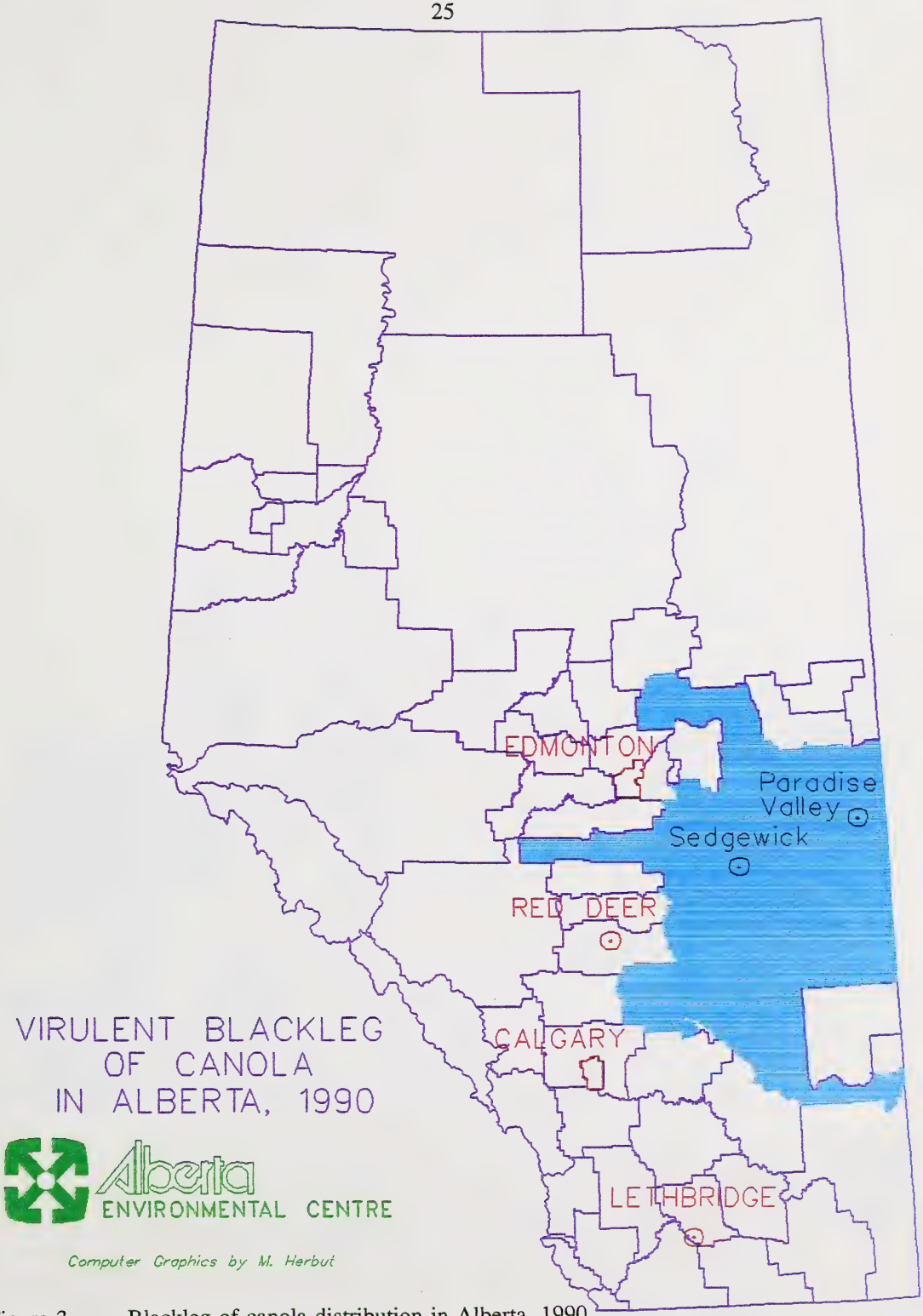


Figure 3. Blackleg of canola distribution in Alberta, 1990.

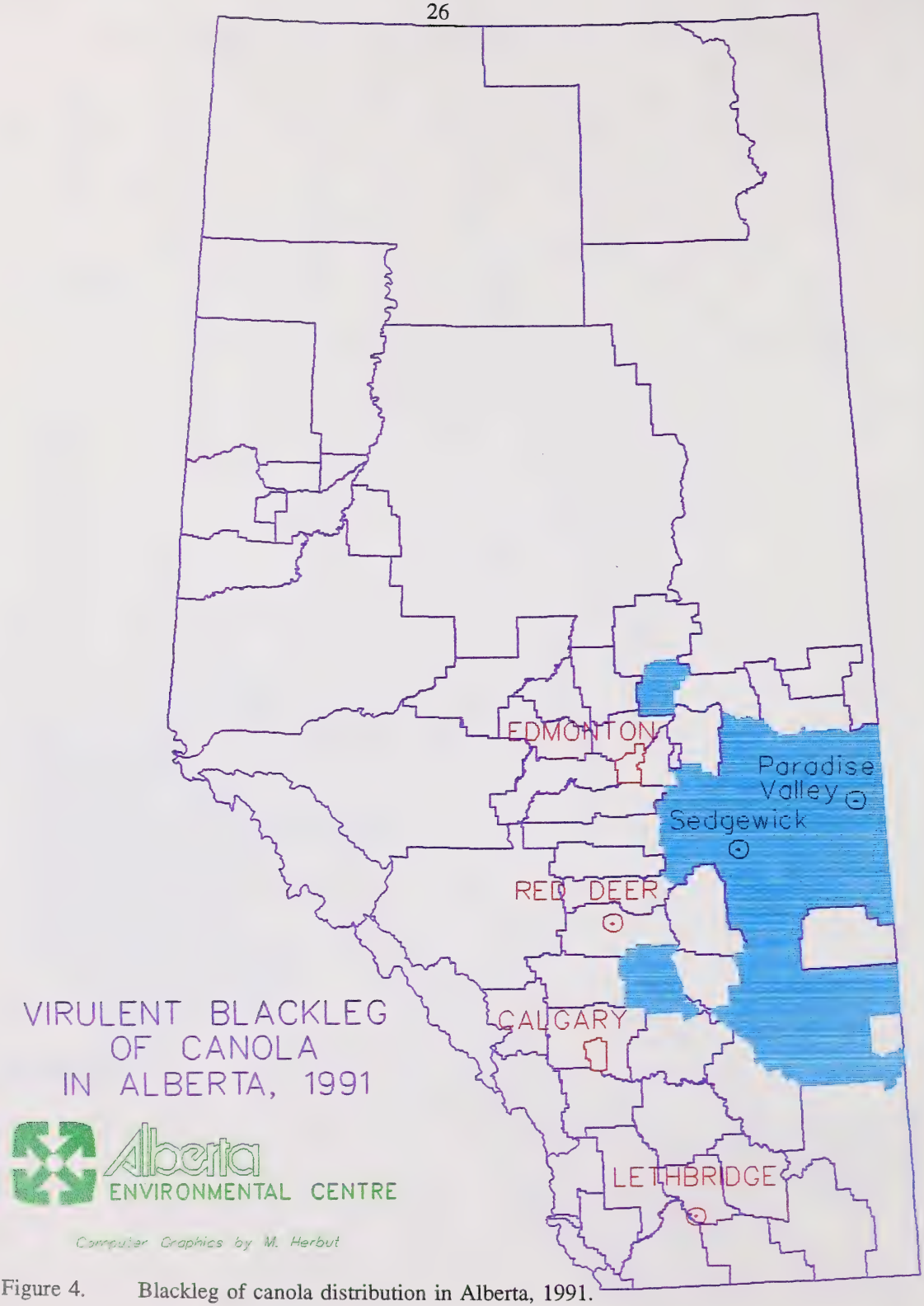


Figure 4. Blackleg of canola distribution in Alberta, 1991.

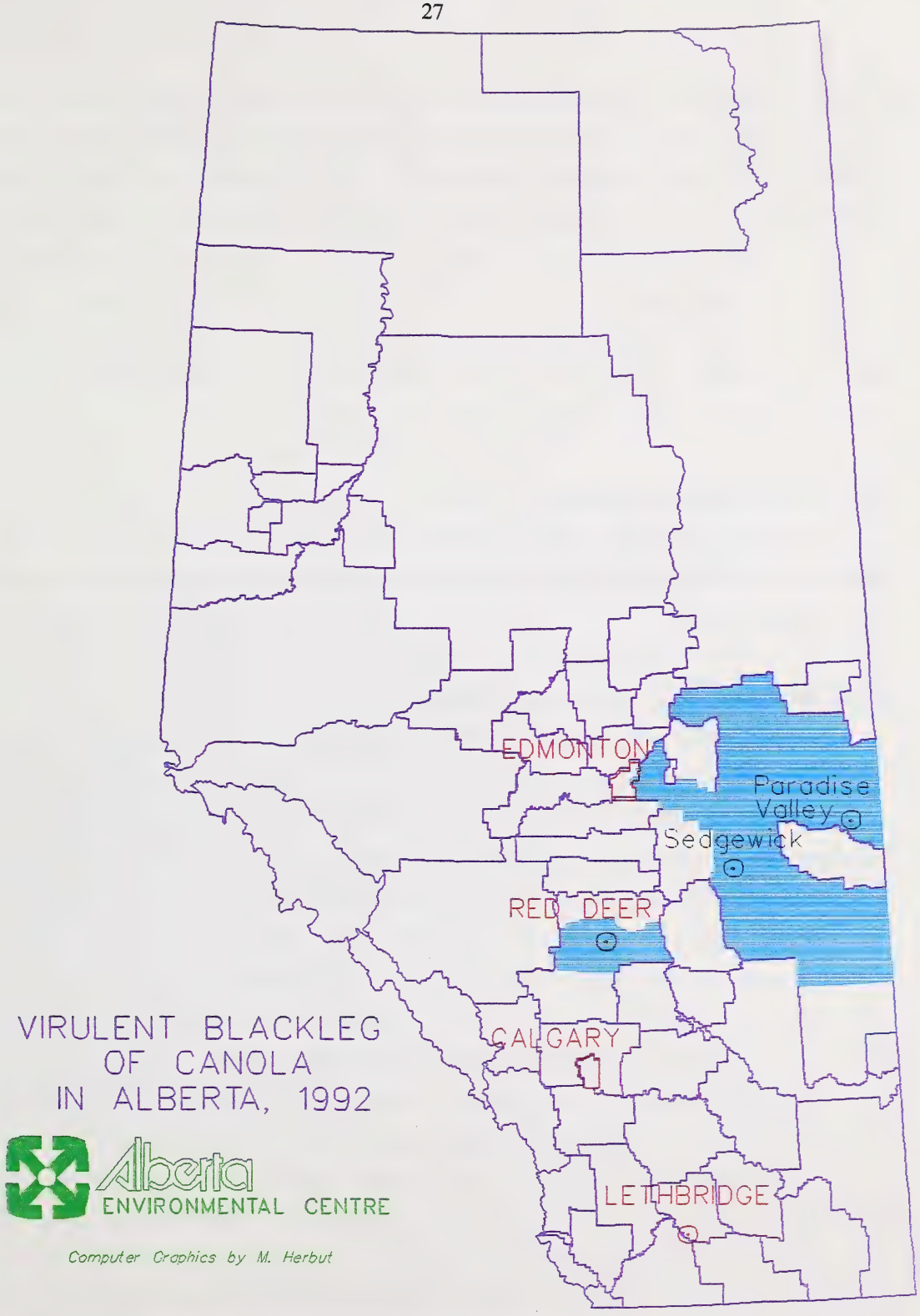


Figure 5. Blackleg of canola distribution in Alberta, 1992.

Centre. The results were quite revealing as percent infected seeds in the infested samples varied between 9 - 23%. This is considerably higher than that found in Saskatchewan. Petrie and Vanterpool (1974) found a maximum seed infection of 6% in 1426 samples tested between 1969 and 1973 and concluded that diseased seed might have contributed significantly to the spread of blackleg into new areas of production. Although the most prevalent strain in Saskatchewan in those years was the weakly virulent strain, the numbers do indicate the explosive nature of the disease. It is conjectured, therefore, that the virulent strain was brought into Alberta with infested seed 2 or 3 years before it was discovered near Vermilion in 1983. To determine the role of ascospores in the disease spread, surveys were started in 1985.

5.3 Survey: Role of Ascospores

To determine the role of ascospores in development of blackleg in Alberta, two studies were carried out: Field surveys to search for pseudothecia and laboratory tests to induce mating of *L. maculans* isolates.

5.3.1 Field Surveys to Search for Pseudothecia

Field surveys of known infected fields in south-central part of the Agriculture Region 4 were started in 1985. Each year, until 1990, stubble samples from crops found infected in the past four years were collected. A minimum of five fields were surveyed for each infection year and a minimum of five basal stem/root pieces per field were examined under the microscope for the presence of pseudothecia and ascospores. No pseudothecia and ascospores were discovered until 1988. In 1989, pseudothecia were suspected on one stubble sample from a crop found infected in 1988 but no ascospores were observed. In 1990, pseudothecia containing immature asci were observed on 1989 crop stubble. In 1991, however, abundant pseudothecia and asci were observed on 1989 crop stubble collected from two separate fields.

The survey results gave clear indication that ascospores were not the source of primary infection in the area surveyed within Agriculture Region 4 until the 1990 crop year.

In a separate field study (details under Chemical control, Section 6.3.2.4), I have been able to trap ascospores using a Burkard spore trap in 1990 and 1991. However, very few spores were trapped between August and October 1989; it is uncertain whether, affected by the environmental conditions, ascospores were not released from pseudothecia or were not present.

Environmental conditions responsible for the development of pseudothecia in the field are not known. The fungus is reported to be heterothallic (Venn 1979), and pseudothecia are formed only when opposite strains of *L. maculans* cross. Laboratory tests were conducted to determine if opposite strains were prevalent in the region heavily infested with blackleg in Alberta.

5.3.2 Laboratory Tests to Induce Mating of *Leptosphaeria maculans* Isolates

Laboratory Tests: Ten highly virulent and five weakly virulent strains of *L. maculans* collected between 1984 and 1989 from various fields distantly located in the infested region (southern half of the Alberta Agriculture Region 4) were crossed. The isolates were crossed with themselves, and with every other isolate using Sach's Agar and sterilized canola stem pieces (Petrie and Lewis 1985). The plates were incubated at 18°C under continuous near-ultraviolet light for 70 days. No ascospores developed in any plate indicating that all isolates used in our studies were most likely of the same strain.

In conclusion, our field surveys and laboratory studies indicate that:

(i) Blackleg was introduced into Alberta most likely with infected canola seed.

(ii) Until 1989, ascospores of *L. maculans* did not play a major role in the disease spread.

(iii) With the discovery of ascospores in Alberta in the past two years, the disease could spread at a faster rate, and the disease control might become more difficult.

Table 7. Cropping history and other features of fields infested with *Leptosphaeria maculans* in north-eastern Alberta, 1984.

Field No.	Cultivar	Seed Source	Seed Treatment	Cropping History (4 yr) 1983	Previous Canola*
Virulent Blackleg Isolated					
OS84-35	Westar	Manitoba (Foundation)	yes	Fallow	1980
OS84-39	Westar	Sask. Wheat Pool	yes	Fallow	1980
OS84-40	Tobin	United Oil Company	yes	Fallow	n/a
OS84-63	Westar	United Grain Growers	yes	Fallow	n/a
Weakly Virulent Blackleg Isolated					
OS84-30	Tobin	Cargill	no	Wheat	1982
OS84-33	Candle	Seed Grower	yes	Wheat	n/a
OS84-41	Tobin	Sask. Wheat Pool	yes	Fallow	1981
OS84-42	Tobin	Lloyd. Wheat Pool	yes	Oats	1981
OS84-49	Westar	United Oilseed	yes	Fallow	n/a

* na: information not available

Table 8. Cropping history and other features of fields infested with *Leptosphaeria maculans* in north-eastern Alberta, 1985.

	Virulent Blackleg Isolated						
					Seed	Cropping history (4 yr)	
Field no.	Incidence	Severity*	Cultivar	Seed source	treatment	1984	Previous canola
11	20	M	Westar	Local Seed Producer	yes	Fallow	not known
12	4	L	Westar	Sand's Seed Farm	yes	Canola	1984
14	5	M	Tobin	Wheat Pool	yes	Fallow	1982
17	20	S	Tobin	United Oilseed	yes	Wheat	1983
20	22	M	Tobin	Wheat Pool	yes	Barley	not known
26	4	M	no information available				
28	5	S	Wheat	Wheat Pool	yes	Fallow	1981
29	16	M	Wheat	Sand's Seed Farm	yes	Fallow	1982
43	4	M	Wheat	Westman Farms	yes	Wheat	1982
44	18	S	Wheat	Westman Farms	yes	Wheat	1982
47	--	--	Triton	D. Jacula	yes	Canola	1984

* Blackleg severity scale: S = Severe; M = Moderate; L = Low.

Table 9. Cropping history and other features of fields infested with *Leptosphaeria maculans* in north-eastern Alberta, 1986.

Virulent Blackleg Isolated						
				Seed treatment	Cropping history (4 yr)	
Field no.	Severity*	Cultivar	Seed source		1985	Previous canola
8	L	Westar	Wheat Pool	yes	Barley	none
9	M	Tobin	Wheat Pool	yes	Barley	1982
12	L	Tobin	Local Grower	1/2	Fallow	none
15	L	Westar	Local Grower	yes	Fallow	1981
18	M	Westar	United Oilseed	yes	Oats	1982
20	M - S	Westar	United Oilseed	yes	Potatoes; Barley	1983
21	M	Westar	Local Grower	yes	Fallow	1983
22	M	Westar	no information available			
24	L	Westar	no information available			
26	M	Tobin	Local Grower	yes	Wheat	1984
27	S	Tobin	Local Grower	yes	Barley	1983
28	L	Westar + Triton	Local Grower	yes	Wheat	---
30	L - M	Westar	Saskatchewan Grower	yes	Barley	1883
31	L	Westar	Local Grower	yes	Barley + Wheat	1976
34	L	Westar	Saskatchewan Grower	yes	Fallow	1976

* Blackleg severity scale: S = Severe; M = Moderate; L = Low.

Table 10. Resistance to blackleg of canola cultivars recommended for Alberta - 1993.

Brassica napus	Blackleg resistance*	*Brassica campestris*	Blackleg resistance*
Westar	5	Tobin	4
Alto	5	AC Parkland	4
Bounty	4	Colt	4
Celebra	3	Eldorado	4
Crusher	3	Goldrush	4
Cyclone	2	Horizon	4
Delta	3	Reward	4
AC Elect	3		
AC Excel	3		
Garrison	2		
Global	2		
HC 120°	4		
Hyola 401	4		
Legend	3		
Profit	3		
Seville	3		
Vanguard	3		
Stallion	3		
AC Tristar	5		

* Disease resistance: 5-highly susceptible; 4-susceptible; 3-moderately susceptible; 2-moderately tolerant; 1-tolerant.

6 DISEASE MANAGEMENT

Since the blackleg-causing fungus is seed-borne and also overwinters in infected stubble, most attempts to control the disease have been targeted at these two sources of primary inoculum.

6.1 Cultural Control

Cultural practices may be the most economical and practical means of controlling blackleg. The isolation of newly sown crops from the previous season's stubble can reduce disease incidence, but this may be of limited practical value because few farmers have their crops sufficiently isolated from neighbours' infested fields to escape severe canker infection initiated with ascospores.

Ploughing infected canola stubble is effective for reducing the amount of debris on the soil surface, and this can apparently reduce the spread of infection to newly sown crops (Gladders and Musa 1980, McGee and Petrie 1979).

A three to four year crop rotation is one of the best methods for controlling the disease because the natural microflora of the soil will destroy the infested stubble and the pathogen. The time required for stubble decomposition, however, depends upon the availability of moisture; micro-organisms are less active under drier conditions. For the crop rotation to be effective, fields should be kept free of volunteer canola and weeds that are susceptible to this disease.

6.2 Resistant Cultivars

All presently grown cultivars of canola in Canada are susceptible to blackleg. Although during the past three years several moderately susceptible cultivars of *B. napus* have been licensed (Table 10), there is an urgent need for developing early-maturing, blackleg tolerant cultivars of *B. napus* and *B. campestris*.

To provide information on blackleg tolerance of the recently licensed canola cultivars under Alberta conditions, research demonstration plots were set up at two farms naturally infested with blackleg, one each in Alberta Agriculture Regions 3 and 4, in the summer of 1992. An interim progress report on this project was submitted to the agencies that funded the research (Kharbanda 1993).

6.2.1 Cultivar Tolerance: Field Evaluation

Two identical experiments were set up at the following two sites found infested with blackleg in 1991:

Sedgewick: NE16 - 45 - 11 - W4

Lloydminster: NW12 - 48 - 1 - W4

Canola cultivars used were:

B. napus - Celebra, Cyclone, Delta, Legend, Westar.

B. rapa - Horizon, Tobin.

All seed was treated with Vitavax RS® at the normal recommended rate (30mL/kg seed) to control seed-borne blackleg, seed decay, seedling blight and flea beetles. Soil was also treated with Furadan (1 g/6 m row) to control flea beetles and aphids.

Canola seeds were planted 2 cm deep in 1.6 m X 6 m plots containing 6 rows, 20 cm apart, at a rate of 200 seeds per row. The experimental design was a split plot with 4 replications. The two main plots consisted of *B. napus* and *B. rapa*. Adequate fertilizer (17-20-0 15% S) was applied with seed as determined by the soil test at each location.

Seeding Dates: Lloydminster- May 26, 1992. Sedgewick- May 19, 1992.

Harvest Dates: Lloydminster- *B. napus*- September 1, 1992; *B. rapa* - August 18, 1992.
Sedgewick- *B. napus*- August 26, 1992; *B. rapa* - August 14, 1992.

To determine yield, middle 4 rows of each plot were trimmed to 5 m, harvested, dried in bags and threshed with a small-plot thresher.

The data on blackleg severity was recorded after harvesting for each plot; the middle four rows were used for disease evaluation. To estimate disease severity, 10 stubbles were pulled from each of the four middle rows, and rated on a scale of 0 - 5. The disease severity scale was: 0 = Healthy, 1 = Trace of blackleg, 2 = up to ⅓ stem girdled, 3 = ⅓ to ⅔ stem girdled, 4 = stem mostly girdled, plant not lodged, 5 = stem girdled, plant lodged.

To determine the effect of blackleg on oil and protein contents of the individual cultivars, the harvested seed from each plot was analyzed for these components.

6.2.2 Cultivar Tolerance: Results and Discussion

6.2.2.1 Cultivar Susceptibility to Blackleg

The blackleg severity was greater, and cultivar reaction to blackleg more clear, at the Sedgewick than at the Lloydminster site. Cyclone was the most tolerant among all the cultivars and developed significantly less disease than in any other cultivar tested at both the locations (Tables 11 and 12). Cultivars Celebra, Delta and Legend were moderately susceptible and did not differ significantly in their reaction to blackleg. On the other hand, Westar was the most susceptible and developed sever stem cankers; at Sedgewick, it developed mean disease severity (MDS) of 4.3, which was significantly higher than in any other *B. napus* or *B. rapa* cultivars (Table 11), resulting in lodging and premature ripening of the plants (Plate 8).

There was no significant difference in disease susceptibility of the two *B. rapa* cultivars, Tobin and Horizon; these cultivars were as tolerant as Legend (*B. napus*) to blackleg (Tables 11 and 12). This is the first report demonstrating that between the two most widely grown cultivars in Alberta, the cultivar Tobin is not as susceptible as Westar to blackleg under field conditions.

6.2.2.2 Cultivar Response in Yield

At Sedgewick, where the disease severity was high, Westar (4 bushels/acre) yielded significantly less than any other cultivar (Table 11, Figure 6). Under practical farming conditions, it would not have been possible to swath such severely affected lodged crop and would have been a total loss. It is noteworthy that Tobin yielded better than Westar under conditions of high disease pressure. There was no significant difference in yields of the rest of the cultivars, however, cultivars Cyclone (30 bushels/acre) and Legend (28.8 bushels/acre) seemed to have performed better than rest of the cultivars.

At Lloydminster, where the disease pressure was low, Westar was one of the highest yielders confirming the high yield potential of this cultivar (Table 12, Figure 7). Celebra, on the other hand, was the poorest yielder. At this site, yield might have been affected by snow that fell in August 1992.

6.2.2.3 Effect of Blackleg on Oil and Protein Content

Results on effect of blackleg on oil and protein contents in seed of the cultivars tested are presented in Tables 13 and 14. There was no significant difference between cultivars in these two characters. In the absence of completely disease-free comparative plots for each cultivar, It is not certain how much oil or protein loss occurred due to blackleg. However, using the long-term average percent oil content of Westar (44.3), and the average units by which each other cultivar differs from Westar, as reported in Western Co-op Tests for canola candidate cultivars conducted by the Western Canada Canola/Rapeseed Recommending Committee, deviation between the expected values for each cultivar and the percent oil obtained in our tests at both the locations was determined. The data was also analyzed using a simple linear regression procedure (Figure 8). The results show that deviation from expected percent oil contents had significant negative linear relationship with the disease severity and could be expressed by the equation $Y = 0.1150 - 0.2511\ X$, when X represent disease severity on a scale of 0-5. The results confirm earlier suspicions that premature ripening in blackleg affected plants might have negative impact on oil contents of seed. The results reveal that the loss in percent oil increases with the increase in blackleg disease severity.

In conclusion, Cyclone was the most tolerant to blackleg among the canola cultivars tested. Under heavy disease pressure, Cyclone and Legend provided good yield and disease resistance, whereas, Westar was extremely susceptible. However, when blackleg pressure was low, Westar yielded as good as Cyclone and Legend. Compared with Westar, Tobin developed significantly less blackleg. There was a significant negative correlation between blackleg disease severity and loss in percent oil content of seeds. These conclusions are, however, based on one year's research and need to be confirmed for at least one more year.

Table 11. Performance of *Brassica napus* and *Brassica rapa* cultivars in a blackleg-infested field near Sedgewick, 1992.

Cultivar	Mean disease severity*	Mean yield (grams/plot)*	Bushels/ acre	Oil %*	Protein %*
Brassica napus					
Celebra	2.612 B	451.1 A	20.1	43.44 A	26.71 A
Cyclone	1.175 C	646.6 A	28.8	43.16 A	26.16 A
Delta	2.325 B	481.0 A	21.5	43.01 A	26.12 A
Legend	2.025 B	663.5 A	30.0	43.31 A	26.34 A
Westar	4.325 A	89.2 B	4.0	43.01 A	25.34 A
Brassica rapa					
Horizon	2.300 B	206.9 B	9.2	42.47 A	24.21 A
Tobin	2.350 B	232.6 B	10.4	42.13 A	24.08 A

* Mean of four replications; mean in columns followed by the same letter do not differ significantly according to Duncan's Multiple Range Test, (P=0.05).

Table 12. Performance of *Brassica napus* and *Brassica rapa* cultivars in a blackleg-infested field near Lloydminster, 1992.

Cultivar	Mean disease severity*	Mean yield (grams/plot)*	Bushels/ acre	Oil %*	Protein %*
Brassica napus					
Celebra	1.350 B	385.2 C	17.2	43.55 A	27.48 A
Cyclone	0.700 C	517.0 ABC	23.1	43.23 A	26.77 AB
Delta	1.362 B	541.4 AB	24.2	43.16 A	26.24 B
Legend	1.237 B	540.6 AB	24.1	43.53 A	27.70 A
Westar	2.900 A	627.2 A	28.0	43.59 A	27.29 A
Brassica rapa					
Horizon	1.200 B	399.4 BC	17.8	42.44 A	25.86 A
Tobin	1.237 B	367.7 C	16.4	42.47 A	25.86 A

* Mean of four replications; mean in columns followed by the same letter do not differ significantly according to Duncan's Multiple Range Test, (P=0.05).

Plate 8. Performance of *Brassica napus* (A) and *Brassica rapa* (B) cultivars in a blackleg infested field, 1992. Note premature ripening and lodging in Westar severely affected with blackleg.

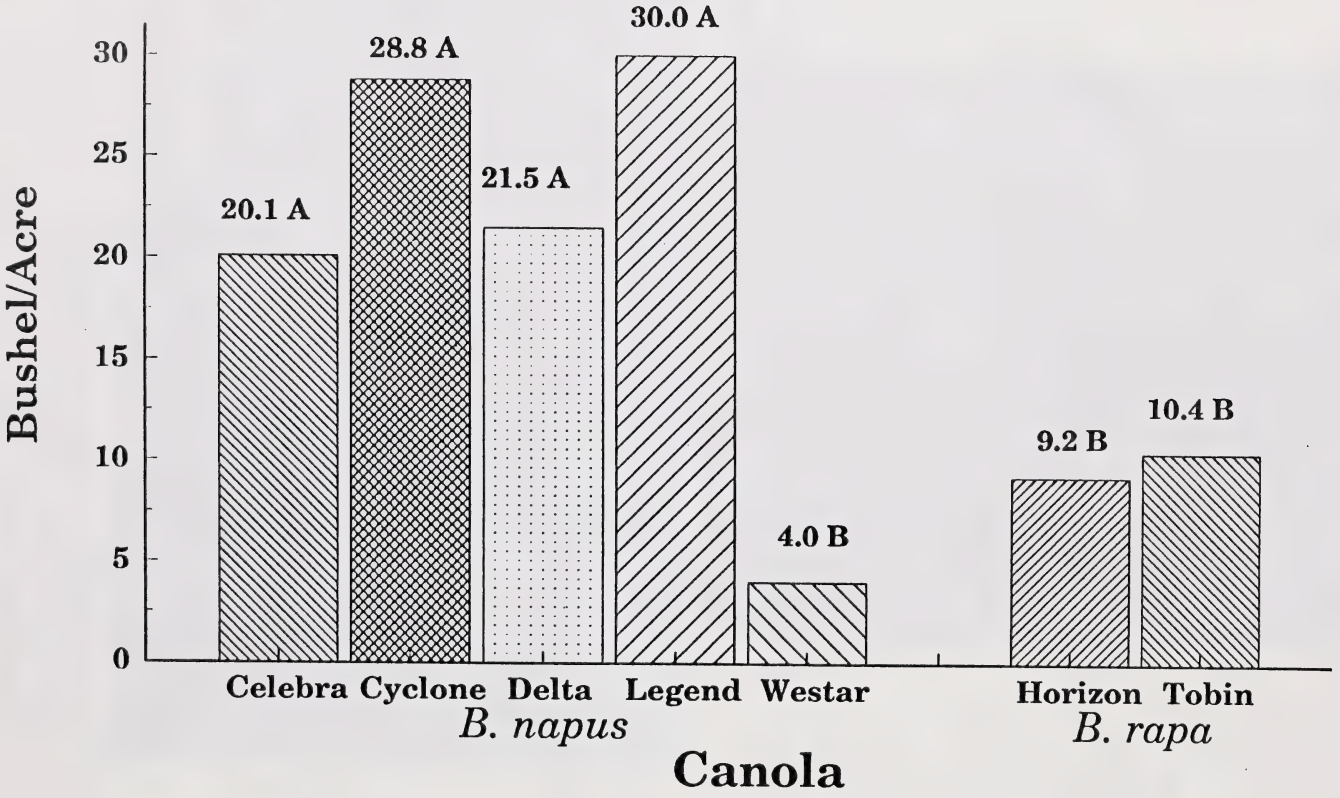


Figure 6. Yield response of *Brassica napus* and *Brassica rapa* cultivars in a blackleg-infested field near Sedgewick, 1992.

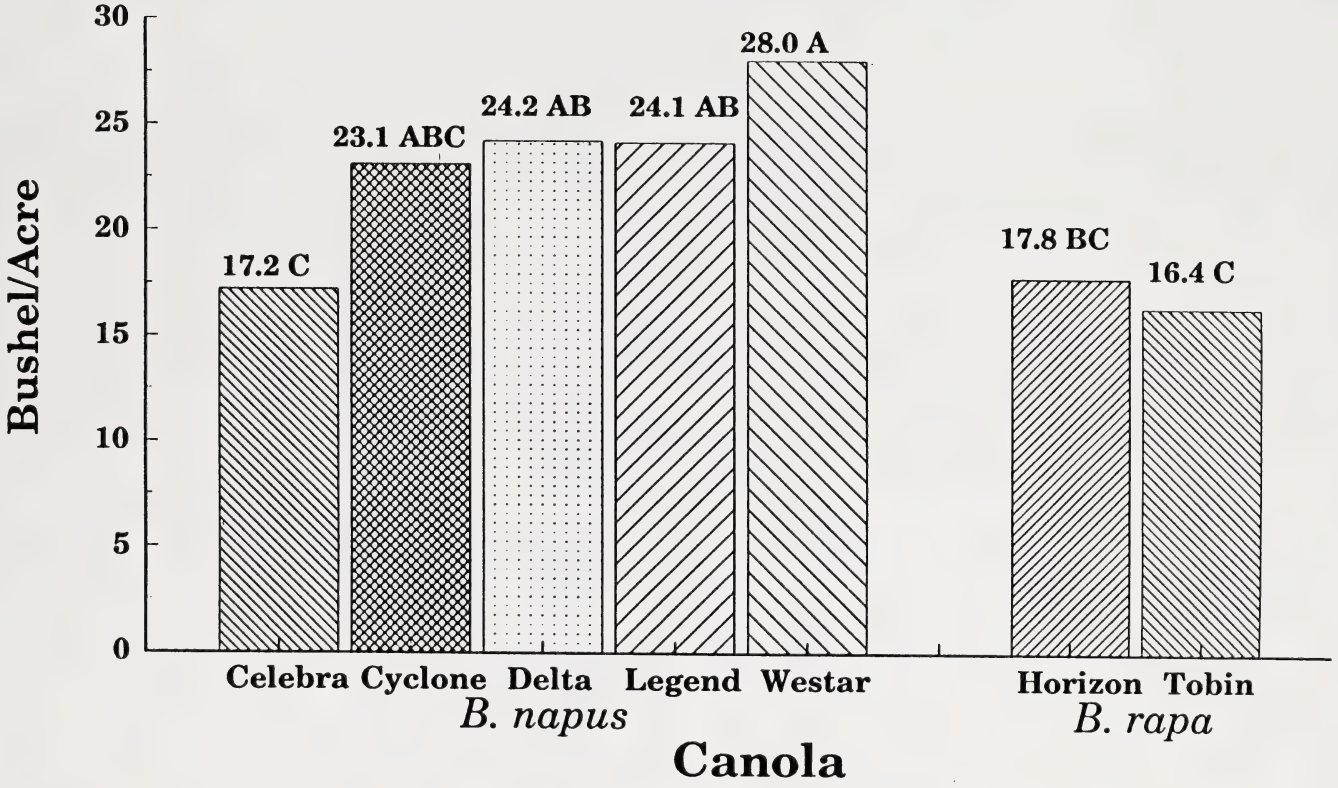


Figure 7. Yield response of *Brassica napus* and *Brassica rapa* cultivars in a blackleg-infested field near Lloydminster, 1992.

Table 13. Effect of blackleg disease severity on percent oil contents in canola seeds of several *Brassica napus* cultivars grown at Sedgewick, 1992.

Cultivar	Mean disease severity*	Reported % oil	Observed % oil*	Deviation from reported % oil
Brassica napus				
Celebra	2.612	43.6	43.44	- 0.16
Cyclone	1.175	43.7	43.16	- 0.54
Delta	2.325	43.5	43.01	- 0.49
Legend	2.025	43.3	43.31	+ 0.01
Westar	4.325	44.3	43.01	- 1.29
Brassica rapa				
Horizon	2.300	43.2	42.47	- 1.74
Tobin	2.350	41.9	42.13	+ 0.23

* Mean of four replications.

Table 14. Effect of blackleg disease severity on percent oil contents in canola seeds of several *Brassica napus* cultivars grown at Lloydminster, 1992.

Cultivar	Mean disease severity*	Reported % oil	Observed % oil*	Deviation from reported % oil
Brassica napus				
Celebra	1.350	43.6	43.55	- 0.05
Cyclone	0.700	43.7	43.23	- 0.47
Delta	1.362	43.5	43.16	- 0.34
Legend	1.237	43.3	43.53	+ 0.23
Westar	2.900	44.3	43.59	- 0.71
Brassica rapa				
Horizon	1.200	43.2	42.44	- 0.56
Tobin	1.237	41.9	42.47	+ 0.57

* Mean of four replications.

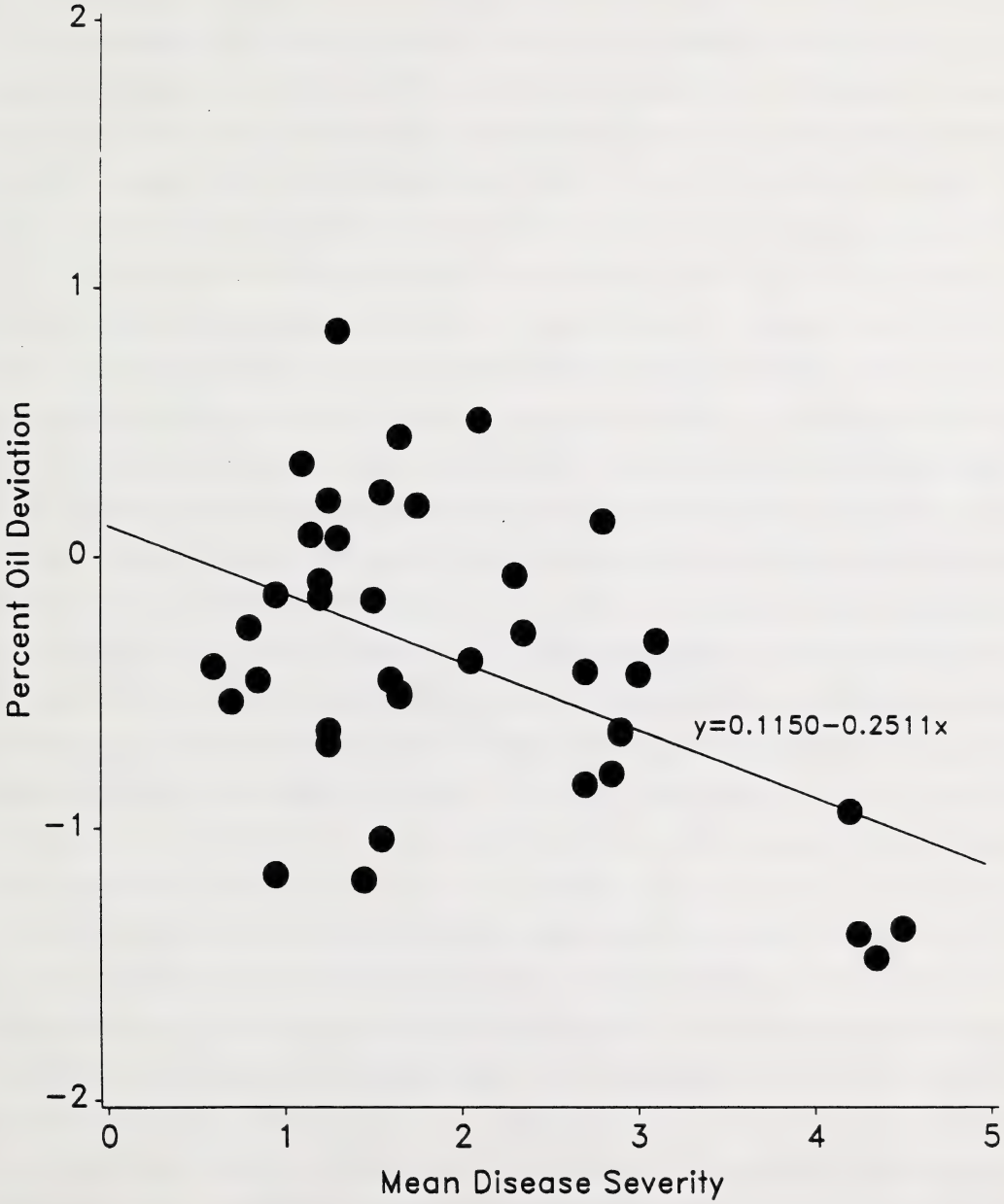


Figure 8. Regression of percent oil deviation from reported values and mean disease severity in five *Brassica napus* cultivars, grown at two locations, 1992.

6.3 Chemical Control

Since the incidence of serious crop damage due to blackleg has increased steadily in recent years (Hopkin *et al.* 1989, Jesperson 1989, Kharbanda *et al.* 1989, Petrie 1985, Platford & van den Berg 1989), it has generated demand to control the disease with fungicides. Though some fungicidal seed-slurry and seed-soak treatments have been employed successfully to control seed-borne *L. maculans* (Gabrielson *et al.* 1977, Jacobsen & Williams 1971, Maude *et al.* 1984), seed treatments have failed to control the disease under field conditions (Barbetti 1975; Brown *et al.* 1976, McKenzie & Verma 1988, Rawlinson & Muthyalu 1979). In Canada, there are no foliar fungicides registered to control blackleg. Since canola cultivars resistant to the disease are not available for commercial use, research was initiated to develop fungicidal control of the disease by combining seed treatments with promising foliar fungicides and partial crop resistance to blackleg available in some recently licensed cultivars. Also, the role of ascospores as primary inocula to initiate development of blackleg of canola in spring has not been previously investigated in Alberta. In this province, ascospore-producing fruiting bodies of the fungus, pseudothecia, were not found until 1989. It is commonly believed that early spring infections are initiated by ascospores and pycnidiospores. Recent studies in Saskatchewan show that the fungus produces ascospores on canola stubble as early as June, about 10 months after crop harvest. However, it is not known when, under natural conditions, the ascospore population in the environment is at its peak during the season. This information would assist in establishing proper timing for the fungicidal spray which should coincide with the earliest heavy build-up of ascospores when the environmental conditions for the disease development are also favourable.

The initial objectives of this study were: (i) to determine the length of time seed treatment fungicides provide protection against blackleg to the emerging seedlings, and (ii) to determine protective and curative effects of fungicidal sprays against blackleg. Experiments were conducted in controlled environments and in the field between 1986 and 1989. From all fungicides evaluated, two of the most promising ones were selected for further field-testing in 1990 and 1991, and the study was expanded to include the following objectives: (iii) to determine the most suitable timing of fungicidal foliar sprays to maximize control, and (iv) to integrate fungicidal control of blackleg with resistance to the disease in a moderately susceptible canola cultivar. Since the timing of a fungicidal spray could be affected by the number of ascospores of *L. maculans* present in the environment, their concentration was monitored with

a spore trap in 1990 and 1991. A report was submitted to the agencies that funded the research (Kharbanda 1992b). A part of the research results have been published (Kharbanda 1992a).

To determine the protective and curative action of fungicides, several fungicides were screened in preliminary petri-plate tests for toxicity towards *L. maculans* conidia and only those found to be toxic were evaluated further as a seed treatment, foliar spray or both. All laboratory and growth chamber tests were repeated at least once.

A single-spore isolate of a highly virulent strain of *L. maculans*, BL-A, recovered from naturally infested plants in Alberta, was used. Plants were inoculated with a conidial suspension prepared in 0.5% gelatin solution.

The planting medium used in growth chamber tests was a pasteurized soil mix containing peat:sand:soil (1:1:1, by volume) in 15-cm-diameter fibre pots. To ensure high humidity for seedling infection, the pots were watered before inoculation and covered individually with plastic bags for 48 hours after inoculation. The light and temperature regime in the growth chamber was 16 hours light/21°C and 8 hours dark/15°C. Light intensity was 300 $mEm^{-2}s^{-1}$ provided by cool white fluorescent tubes and incandescent lamps.

Unless specified otherwise, *B. napus* cv. Westar was used. This and *B. rapa* cv. Tobin have been widely grown in western Canada and are highly susceptible to blackleg. Westar generally develops a heavier canopy than does Tobin, and therefore is probably more susceptible to infection under field conditions.

6.3.1 Seed Treatments

The fungicides used for seed treatment studies were: benomyl (Benlate® 50 WP, DuPont); carbathiin + thiram (UBI-2390-2, 33.3 FL, Uniroyal); iprodione (Rovral® ST 16.7 FL, Rhone-Poulenc); prochloraz (Sportak® 20 SN, Elanco); propiconazole (Tilt® 25 EC, Ciba-Geigy), thiabendazole (Mertect® 45 FL, Chipman); and tolclofos-methyl (Rizolex® 50 WP, Sandoz).

Seed of cvs. Tobin and Westar was inoculated with *L. maculans* following a modified method of Maude *et al.* (1984). The seed was surface-sterilized by soaking in a 1.0% solution of NaOCl for 5 minutes, rinsed once with sterile distilled water, and then soaked for 18 hours in a conidial suspension (10^7 conidia/mL) three times its volume. The soaked seeds were spread

on a sterile cheesecloth to dry under a fumehood for 24 hours, and stored in paper bags at 4°C until used. The seed infestation was nearly 100%.

The inoculated or healthy seed was treated with individual fungicides at the manufacturers' recommended rates in batches of 100 gm in 500-mL erlenmeyer flasks. The flasks were agitated briskly to obtain uniform coverage of the seed. The efficacy of seed treatments was evaluated in laboratory, growth chamber and field tests.

6.3.1.1 Laboratory Tests

Agar plate tests were conducted to determine the direct effect of seed treatments on the seed-borne fungus and to select the more effective ones for further work. Fifty artificially infested seeds of the two cultivars, untreated or treated with one of the fungicides, were plated on potato dextrose agar (PDA) amended with streptomycin sulfate (150 mg/L) in 90-mm-diameter petri-plates at 10 seeds per plate. The plates, arranged in a completely randomized design with five replications, were incubated at 21°C. After 7 days incubation in the dark, the plates were irradiated with near-UV lights for 16 hours per day to promote pycnidial production. The number of *L. maculans* colonies on each plate was recorded after 21 days. The experiment was repeated once, and the data were pooled. Since the percentage data varies between 0-100, arcsin transformation was done prior to analysis of variance. The experiment was analyzed as 7 (treatments) x 2 (cultivars) factorial.

6.3.1.2 Growth Chamber Tests

These were conducted to study protection against *L. maculans* conferred to young emerging seedlings by fungicidal seed treatments. A modified method of Helms & Cruickshank (1979) was followed: 25 healthy seeds of cv. Westar, untreated or treated with one of the fungicides, were planted in the pasteurized soil mix. The seeds were placed on the moistened surface of the soil mix and covered with 150 mL perlite mixed with 50 mL conidial suspension ($5x10^6$ conidia/mL). The seeded pots were covered individually with plastic bags to maintain high humidity for four days. All treatments were replicated four times and arranged in a completely randomized design in a growth chamber. The number of infected seedlings (with typical pycnidia) in each pot were recorded at various time intervals until 28 days after seeding.

6.3.1.3 Field Tests

The experiments were carried out during the summers of 1988 and 1989. Healthy seeds of cv. Westar, untreated or treated with one of the fungicides, were planted in 6 m x 0.9 m plots, replicated four times in a randomized complete block design. Each plot was divided into four quadrants and seedlings in one of the quadrants in each plot were inoculated by spraying with a conidial suspension of *L. maculans* ($5x10^6$ conidia/mL). Inoculation was done 21 days after seeding in 1988, or 15 days after seeding in 1989. Immediately after inoculation, the entire quadrant was covered with a plastic sheet which was pegged to the ground to maintain high humidity and removed early next morning. A second quadrant in each plot was inoculated 28 days after seeding in 1988, and 21 days after seeding in 1989. Infected seedlings were detected in the first quadrants when observed 12 days after inoculations each year. Since the fungicides did not appear to protect the seedlings from infection, inoculation of the remaining two quadrants was considered unwarranted and the quadrants were abandoned.

6.3.1.4 Results: Seed Treatments

In agar plates, all the fungicides effectively inhibited seed-borne *L. maculans* in both Westar and Tobin. Prochloraz was the most effective fungicide and completely prevented fungus recovery from infected seeds of both cultivars (Table 15). The ANOVA of this factorial experiment (Table 16) revealed that fungicide x cultivar interaction was highly significant. The interaction obviously was due to the decrease in effectiveness of thiabendazole and tolclofos-methyl on Tobin relative to Westar; the interaction were not significant when the data were analyzed without these two fungicidal treatments.

In the growth chamber test, iprodione and prochloraz effectively protected the seedlings from blackleg infection up to 15 days after seeding in infested perlite (Table 17); benomyl and carbathiin + thiram were the only other treatments that protected more than 50% of seedlings from infection. Twenty-one days after seeding, iprodione was the only treatment that prevented infection in more than 90% of the seedlings; prochloraz was the next most effective with 45% healthy seedlings. On the 28th day, almost all seedlings in the check were infected and the percentage of healthy seedlings for iprodione and prochloraz declined to 38 and 22, respectively, indicating that the effectiveness of these fungicides was also reduced considerably.

In the field test, none of the seed treatments prevented emerged seedlings from infection when inoculated with *L. maculans* conidia 21 days after seeding in 1988, or 15 days after seeding in 1989. All inoculated seedlings in each fungicide-treated and check plot were infected.

Table 15. Inhibition by fungicidal seed treatments of *Leptosphaeria maculans* growth from artificially infected canola seed on potato dextrose agar + streptomycin medium 21 days after incubation at 21°C.

Fungicide	Rate (g, a.i./kg seed)	% *L. maculans* colonies	
		cv. Westar	cv. Tobin
Benomyl	2.0	0	6
Carbathiin + thiram	1.0 + 2.0	2	4
Iprodione	5.0	6	4
Prochloraz	2.5	0	0
Thiabendazole	2.0	0	26
Tolclofos-methyl	3.0	16	52
Check		100	100

Table 16. Analysis of variance of percent *Leptosphaeria maculans* colonies data presented in Table 15.

Source	Degree of Freedom	Sum of Squares	F-ratio
Cultivars	1	647.81	35.02**
Fungicides	6	28938.97	260.73**
Fungicides x Cultivars	6	1014.52	9.14**
Error	14	258.99	
Total	27	30860.29	

** Significant at P=0.01; analysis was performed on arcsin-transformed data.

Table 17. Mean percent healthy seedlings of canola cv. Westar from seed treated with different fungicides and planted in a soil mix covered with perlite infested with *Leptosphaeria maculans* in a growth chamber.

Fungicide	Rate (g, a.i./kg seed)	Percent Healthy Seedlings* Days after seedings		
		15	21	28
Benomyl	2.5	52 bc	22 c	11 c
Carbathiin + thiram	1.0 + 2.0	64 b	19 c	10 c
Iprodione	5.0	100 a	91 a	38 a
Prochloraz	2.5	99 a	45 b	22 b
Propiconazole	0.025	8 d	3 d	3 c
Tolclofos-methyl	3.0	46 c	7 d	2 c
Check		10 d	5 d	1 c

* Means within a column followed by the same letter do not differ significantly (P = 0.05) according to Duncan's Multiple Range Test. Analyses performed on arcsin-transformed data; values in the columns were back-transformed after analyses.

6.3.2 Foliar Sprays

Some of the fungicidal formulations evaluated were the same as for the seed treatments. Others were: cyproconazole (SAN-619F 100 SL, Sandoz); ethyltrianol (HWG-1608 3.6 FL, Chemagro); iprodione (Rovral® 25 FL, Rhone-Poulenc); mancozeb (Manzate-200® WP, DuPont); prochloraz (Sportak® 40 EC, Elanco). An adjuvant was added to each fungicide except Rovral 25 FL, which contains a surfactant in its formulation. The adjuvant used with prochloraz was Enhance® (Fatty amine ethoxylate 78%, Elanco) 0.1% (v:v), and with all other formulations it was Agral 90® (nonylphenoxy polyethoxyethanol 90%, Chipman) 0.1% (v:v) in 1987, 1988, and Assist® (an oil concentrate, BASF) 1.0% (v:v) in 1989. Experiments were conducted in the growth chamber and in the field.

6.3.2.1 Growth Chamber Tests

Untreated, healthy seed of cv. Westar was planted in pasteurized soil mix in fibre pots. Seedlings were thinned to five per pot after emergence.

To determine protective action of the test fungicides, 6-week-old seedlings were sprayed with a conidial suspension to the point of run-off four days prior to foliage inoculation. Each fungicide was also evaluated as two sprays; the second spray was applied six days after inoculation. The fungicide treatments were replicated four times and the pots arranged in a completely randomized design in the growth chamber. The disease severity (DS) was evaluated for leaves and stems four weeks after the fungicides were sprayed.

To evaluate DS on leaves, a scale was developed based on extent of leaf and petiole infection and presence of pycnidia: 0 = no lesions; 1 = no leaf wilted, one or more leaves/petioles with small lesions up to 1 mm in diameter, pycnidia absent; 2 = one leaf wilted or severed from stem, other leaves with small lesions without pycnidia; 3 = up to three leaves wilted, other leaves with small lesions without pycnidia; 4 = up to three leaves wilted, several other leaves with lesions and pycnidia; 5 = extensive defoliation; lesions with pycnidia present at leaf scars.

The DS on stems was evaluated using the scale: 0 = no infection, 1 = small infected lesion, no pycnidia; 2 = lesion less than 1/3 of the stem diameter, pycnidia present; 3 = lesion up to 2/3 of the stem diameter, plant not wilted; 4 = stem mostly girdled, plant not wilted; 5 = stem mostly girdled, tissue damage extensive, plant wilted.

All five plants in a pot (replication) were rated individually and mean disease severity (MDS) was calculated using the formula:

$$\text{MDS} = \frac{\sum (\text{no. of plants in category} \times \text{DS numerical value})}{\text{Total number of plants rated}}$$

To determine curative actions of fungicides, seedlings were sprayed either once, four days after stem inoculation, or twice, four and 10 days after inoculation. To inoculate, a one-mm mycelial plug from an actively growing *L. maculans* culture on PDA was inserted in a slit made with a sterile scalpel at the base of a 6-week-old seedling. The wound was then covered with a wet cotton plug and wrapped with parafilm to provide high humidity. The parafilm was removed after 72 hours. The check plants were subjected to the same procedure without insertion of the fungus.

The DS was evaluated for stems and MDS was calculated as described for foliage inoculation experiments.

The experiments were analyzed as 6 (main treatments) x 2 (# of sprays) factorials. The disease severity data on stem and leaf were analyzed separately by analysis of variance. When fungicide x spray interaction was not significant, Duncan's multiple range test was used to separate means of different treatments.

6.3.2.2 Field Tests

These were conducted with either artificial inoculation or natural infestation. All field plots were 6 m x 0.9 m consisting of four rows 15 cm apart; the treatments were replicated four times in a randomized complete block design. Plots were separated from each other with a 0.9 m-wide strip of barley to reduce interplot interference.

In 1988, the plots were inoculated once at the four-leaf stage, growth stage (G.S.) 2.4 of Harper and Berkenkamp (1975). In 1989, two experiments were conducted: plots in one experiment were inoculated twice, once at G.S. 2.3 and again seven days after the fungicides were sprayed (G.S. 2.5). The other experiment was conducted in a naturally infested field and was not artificially inoculated.

Fungicides were sprayed at the manufacturers' recommended rates with a knapsack sprayer. In the experiment in 1988, the fungicides were sprayed once only at the bud stage (G.S. 3.1). In 1989, in both experiments fungicides were sprayed either early (G.S. 2.5), or early and late (G.S. 4.1). Check plots were sprayed with water.

6.3.2.3 Results: Foliar Sprays

In the growth chamber test, fungicide x spray interaction was significant only for the data from inoculated leaves (Tables 18, 19, 20, 21). A graph between MDS on leaves due to various fungicides vs. numbers of sprays revealed that the interaction was probably due to the check treatment (water sprays) (Figure 9). This interaction disappeared when the data were analyzed excluding the check indicating that fungicidal effects were independent of the number of sprays: the overall trend was a decrease in mean disease severity with a second spray application.

Significant disease reduction was achieved in stem-inoculated plants by a single spray for all treatments except benomyl which was effective only when sprayed twice (Table 18).

Ethyltrianol and propiconazole were, however, phytotoxic at the rates evaluated, and caused stunting, leaf scorching and puckering. Plants sprayed even once with either iprodione or prochloraz looked vigorous and healthy (Plate 9). Generally, the reduction in DS with two sprays was not significantly improved relative to that achieved with one spray. Similar results were obtained in foliage-inoculated plants. The untreated, inoculated check plants were severely defoliated and eventually developed basal stem cankers, whereas those treated with iprodione or prochloraz were healthy and had minimal disease on either leaves or stems (Table 18).

In the 1988 field test, under artificial inoculation, ethyltrianol and prochloraz used with an adjuvant were the most effective treatments (Table 22). These treatments resulted in significantly more healthy plants and better grain yield than in the check. Considerable variation occurred among replications of the iprodione treatment and some plots looked as healthy as those treated with ethyltrianol + adjuvant or prochloraz + adjuvant. Propiconazole + adjuvant did not control the disease but increased yield significantly. Use of an adjuvant improved the performance of prochloraz and propiconazole, the only two fungicides that were also tested without an adjuvant. Benomyl was the least effective fungicide and did not significantly increase the number of healthy plants nor increase yield. Ethyltrianol and propiconazole which were phytotoxic in growth chamber test were less phytotoxic in the field. Some leaf scorching and puckering was evident at an early stage of crop development but the injury declined as the crop aged and was not noticeable in adult plants.

In 1989, when the plots were inoculated twice, prior to and seven days after the first fungicide application, two sprays of prochloraz + adjuvant significantly increased the number of healthy plants over the untreated check (Table 23). Among other treatments, however, no statistically significant differences were observed.

Under conditions of natural infection in 1989, neither iprodione nor prochloraz + adjuvant were effective, and no significant disease reduction or yield increase over the check was obtained; since there were no significant differences, data are not presented.

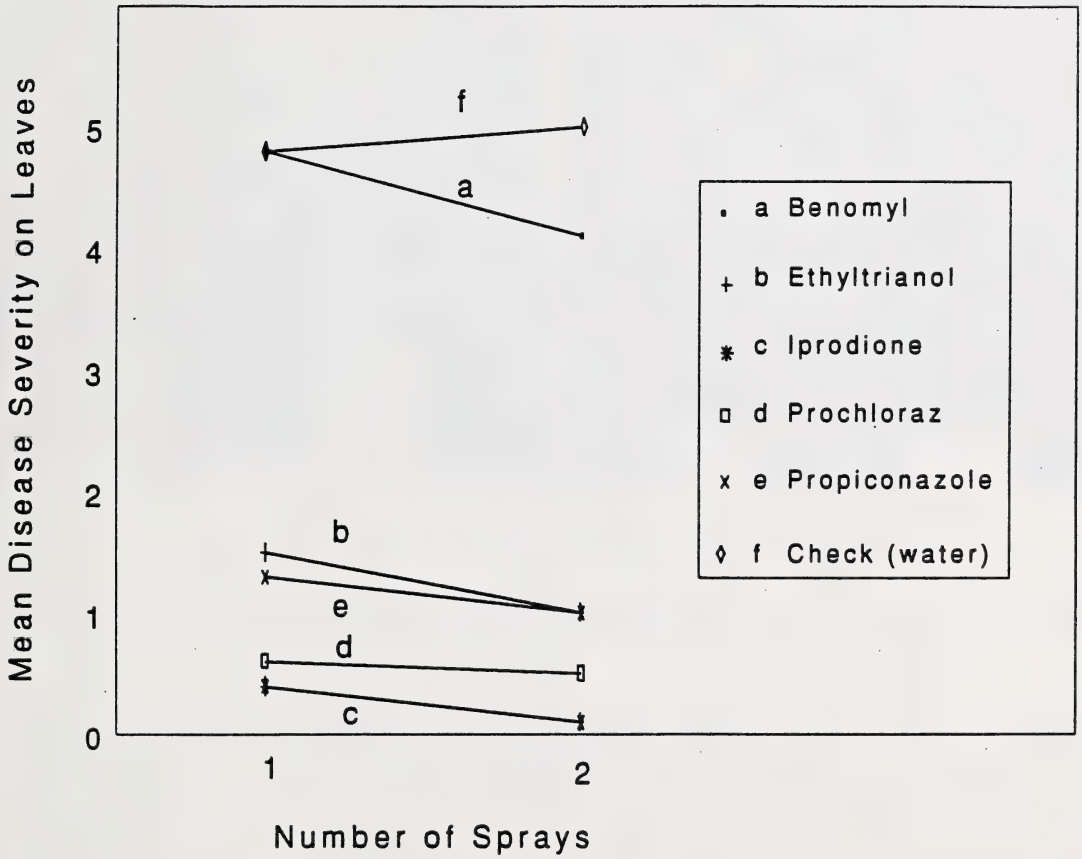


Figure 9. Mean disease severity on leaves inoculated with *Leptosphaeria maculans* conidia and sprayed with a fungicide once, 4 days before inoculation or twice, 4 days before and 6 days after inoculation.

a. b.

Plate 9. Effectiveness of iprodione (Rovral) (a), and prochloraz (Sportak) (b), sprayed on 6-week old canola plants 4 days after stem inoculation with *Leptosphaeria maculans*.

Table 18. Blackleg severity on canola plants cv. Westar following artificial inoculation of stem or leaves and application of fungicidal foliar sprays in a growth chamber.

			Mean Disease Severity (Max. 5)†		
	Rate	Number	Stem inoculation	Foliage inoculation	
Fungicide	(g a.i./L)	of sprays	$Stem_{(i)}$	Leaves	$Stem_{(ii)}$
Benomyl	0.6	1	3.9 ab	4.8 a	2.5 b
		2	3.6 b	4.1 b	2.4 b
Ethyltrianol	0.3	1*	2.1 de	1.5 c	0.5 cd
		2*	1.9 de	1.0 de	0.9 c
Iprodione	0.6	1	2.0 de	0.4 fg	0.1 d
		2	1.6 e	0.1 g	0.1 d
Prochloraz	0.6	1	2.3 cd	0.6 ef	0.5 cd
		2	2.1 de	0.5 fg	0.3 d
Propiconazole	0.15	1*	2.7 c	1.3 cd	0.4 d
		2*	2.0 de	1.0 de	0.3 d
Check		1	4.2 a	4.8 a	3.0 a
		2	4.4 a	5.0 a	3.0 a

† Two 6x2 factorial experiments (stem or foliage inoculation) with 6 levels of fungicides and 2 levels of sprays. Data analyzed separately for Stem (i), (ii) and leaves. Means within a column followed by the same letter do not differ significantly (P = 0.05) according to Duncan's Multiple Range Test.

* Phytotoxic.

Table 19. Analysis of variance of blackleg severity on stem (i) data presented in Table 18.

Source	Degrees of Freedom	Sum of Squares	F-ratio
Sprays	1	0.9633	9.17**
Fungicides	5	43.3367	82.55*
Sprays x Fungicides	5	0.7167	1.33NS
Error	36	3.7800	
Total	47	48.7967	

** Significant at P=0.05; NS, not significant.

Table 20. Analysis of variance of blackleg severity on leaves data in Table 18.

Source	Degrees of Freedom	Sum of Squares	F-ratio
Sprays	1	0.9918	13.54**
Fungicides	5	165.6544	452.21**
Sprays x Fungicides	5	1.1393	3.11*
Error	36	2.6375	
Total	47	140.4230	

*, ** Significant at P=0.05 and P=0.01, respectively.

Table 21. Analysis of variance of blackleg severity on stem (ii) data in Table 18.

Source	Degrees of Freedom	Sum of Squares	F-ratio
Sprays	1	0.0300	0.34NS
Fungicides	5	68.8467	156.87**
Sprays x Fungicides	5	0.5200	1.18NS
Error	36	3.1600	
Total	47	72.5567	

** Significant at P=0.01; NS, not significant.

Table 22. Mean number of healthy plants and mean yield per plot in canola cv. Westar artificially inoculated with *Leptosphaeria maculans* at growth stage 2.4 and sprayed with different fungicides at growth stage 3.1 in field in 1988.

Fungicide	Rate (g, a.i./ha)	Mean number of healthy plants*	Mean yield (g)
Benomyl + adjuvant	500	22.0 b	102 cd
Ethyltrianol + adjuvant	250	39.0 a	146 c
Iprodione	500	32.0 ab	157 bc
Prochloraz	500	27.0 ab	159 bc
Prochloraz + adjuvant	500	40.0 a	204 ab
Propiconazole	125	20.0 b	158 bc
Propiconazole + adjuvant	125	33.0 ab	248 a
Check	Nil	21.0 b	78 d

* Mean of 4 replications; means within a column followed by the same letter do not differ significantly (P = 0.05) according to Duncan's Multiple Range Test.

Table 23. Mean number of healthy plants and mean yield per plot of canola cv. Westar artificially inoculated with *Leptosphaeria maculans* at growth stages 2.3 and 3.5, and sprayed with different fungicides at growth stage 2.5 and 4.1 in field in 1989.

Fungicide	Rate (g, a.i./ha)	Number of sprays	Mean number of healthy plants*	Mean yield 1 (g)*
Benomyl + mancozeb	500 + 1800	1	24.0 b	365.2 ab
		2	16.0 b	344.5 ab
Cyproconazole	150	1	23.0 b	392.7 ab
Cyproconazole**	150	1	14.0 b	183.7 b
		2	20.0 b	398.8 ab
Ethyltrianol	125	1	22.0 b	354.0 ab
		2	21.0 b	377.4 ab
Iprodione	500	1	23.0 b	288.0 ab
		2	26.0 ab	461.0 ab
Prochloraz	500	1	25.0 b	363.2 ab
		2	46.0 a	434.3 ab
Propiconzaole	125	1	20.0 b	357.1 ab
		2	34.0 ab	489.6 a
Check	Nil	2	23.0 b	431.5 ab

* Mean of four replications; means within a column followed by the same letter do not differ significantly (P = 0.05) according to Duncan's Multiple Range Test.

** No adjuvant was added.

6.3.2.4 Ascospore Population and Timing Fungicidal Foliar Spray

The fungicide prochloraz, which was found most promising in the laboratory and growth chamber experiments, was further evaluated to determine the most suitable time of spraying for maximum control of blackleg. Experimental procedures and design were the same as described under foliar sprays above. The cultivar used was Legend which is moderately susceptible to blackleg. Each week, from June 13 to July 27, 1990 a set of four plots, one in each block (replication) was sprayed once with prochloraz. A plot received only one spray during the course

of the experiment; unsprayed plots served as checks. The experiment was repeated in 1991; the sprays were applied between June 19 and July 31.

To monitor the *L. maculans* ascospore population in air, a Burkard 7-day volumetric spore trap (Plate 10) was set up on May 1, 1990 and 1991, and operated until October 25 in 1990 and October 7 in 1991. This machine utilizes adhesive-coated plastic tape to draw, under vacuum, deposits from the air on a continuous basis, seven days a week. A new tape was installed once a week, and the one removed cut up and mounted on slides for viewing under microscope to determine the population of ascospores in the field (Plate 11). A seven-day thermograph to check temperature variations and a rain gauge were also set up close to the spore trap to collect supplementary data on environmental conditions that influence ascospore release and blackleg development.

In 1990, significant control of blackleg was achieved with prochloraz fungicide only when canola was sprayed before it was six weeks old (July 6, Table 24). None of the sprays, however, improved yield. The daily spore count data show (Figure 10) that ascospores were caught between May 15 and September 15 but relatively larger numbers of spores were caught within a day or so after there was a precipitation of over 5 mm; the daily maximum temperature on these days was below 15°C.

In 1991, the disease severity was generally higher than in 1990, and spraying of prochloraz any week during the growing season did not cause a significant reduction in mean disease severity or increase yield (Table 25). As well, the spore trap data show that frequency of occurrences of large numbers of ascospores was greater in 1991 than in 1990 (Figures 10 & 11).

6.3.2.5 Integration of Fungicidal Control with Disease Resistance

In 1990, seed of a susceptible (Westar) and a moderately susceptible (Legend) cultivar were planted in field plots keeping the procedure and experimental design the same as specified above under foliar sprays. Fungicide, iprodione or prochloraz, was sprayed either once (early, mid-June) or twice (early and late, mid-June and early July) on the two cultivars.

DS and seed yield were determined using all plants in 5-m-sections of the middle two rows of each plot. The DS was evaluated on the scale described for stem infection in growth chamber experiments. To determine disease control achieved due to fungicides, the numbers of

healthy plants for each treatment were compared. Plants with severities of 0, 1 or 2, which have a minimal effect on canola yield (unpublished data), were pooled as healthy plants. To determine yield, plants were hand-pulled and threshed.

The data were analyzed as 2 (cultivars) x 3 (fungicides) x 2 (sprays) factorial. Two separate analyses were done using MDS or number of healthy plants resulting from various treatments (Tables 26, 27, 28, 29; Figure 12); however, the conclusions drawn from the two analyses were essentially the same. ANOVA of blackleg severity data show that none of the various interactions were significant, but the differences within varieties and fungicides were highly significant (Table 27). Further data analysis with Duncan's multiple range test showed that the mean disease severity in unsprayed Legend was significantly less than that in unsprayed Westar confirming that Legend has superior blackleg tolerance. A significant reduction in disease severity occurred in Westar with a single spray of either of the two fungicides. In Legend, which developed considerably less disease than Westar to begin with, iprodione was ineffective and it required two sprays of prochloraz to significantly reduce the disease severity compared with the untreated check (Table 26).

In Westar, two sprays of prochloraz caused a significantly greater reduction in mean disease severity of blackleg than two sprays of iprodione (Table 26). When data for Legend and Westar were pooled, the disease reduction achieved with prochloraz was significantly greater than with iprodione (Table 28; Figure 12).

6.3.3 Chemical Control: Discussion

Studies reported here demonstrate good protective and curative properties of iprodione and prochloraz against *L. maculans* on canola under controlled environmental conditions. When used as seed treatments these fungicides effectively suppressed seed-borne inoculum and also protected seedlings from infection from external sources for about three weeks after seeding in the growth chamber. Benzimidazole fungicides, e.g. benomyl and thiabendazole, were also quite effective for controlling the seed-borne fungus (Table 15), which confirms earlier reports of Brown *et al.* (1976) and Maude *et al.* (1984) on effectiveness of benomyl seed treatments against *L. maculans*. It is noteworthy that benomyl, which showed acute fungitoxicity to seed-borne *L. maculans* (Table 15), was not very effective for protecting seedlings (Table 17).

Plate 10. Burkard 7-day volumetric spore trap (A) and a cage (B) housing a thermograph installed in a blackleg-infested field, 1990.

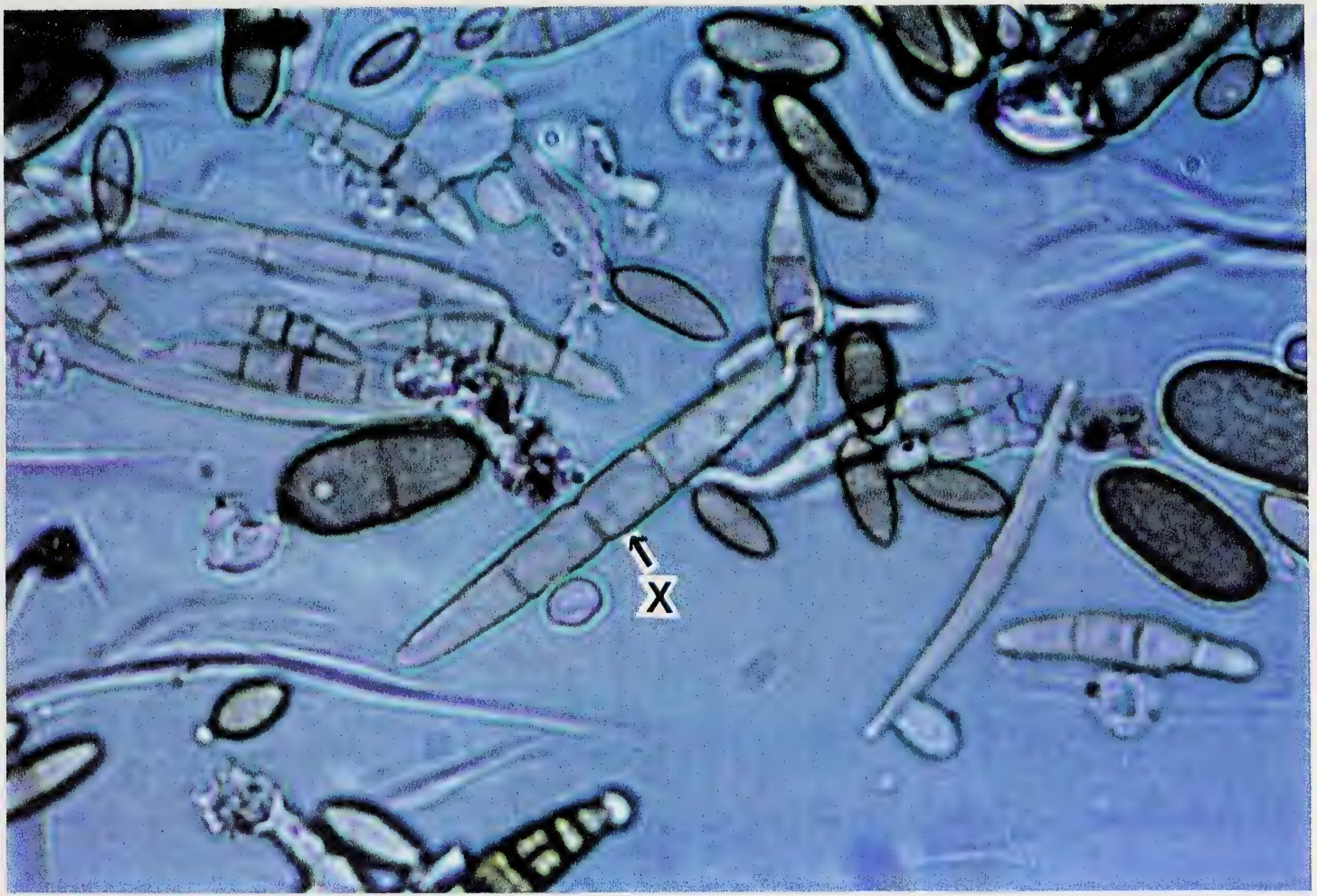


Plate 11. Portion of tape from spore trapping showing deposits of fungal spores including an ascospore (X) of *Leptosphaeria maculans*.

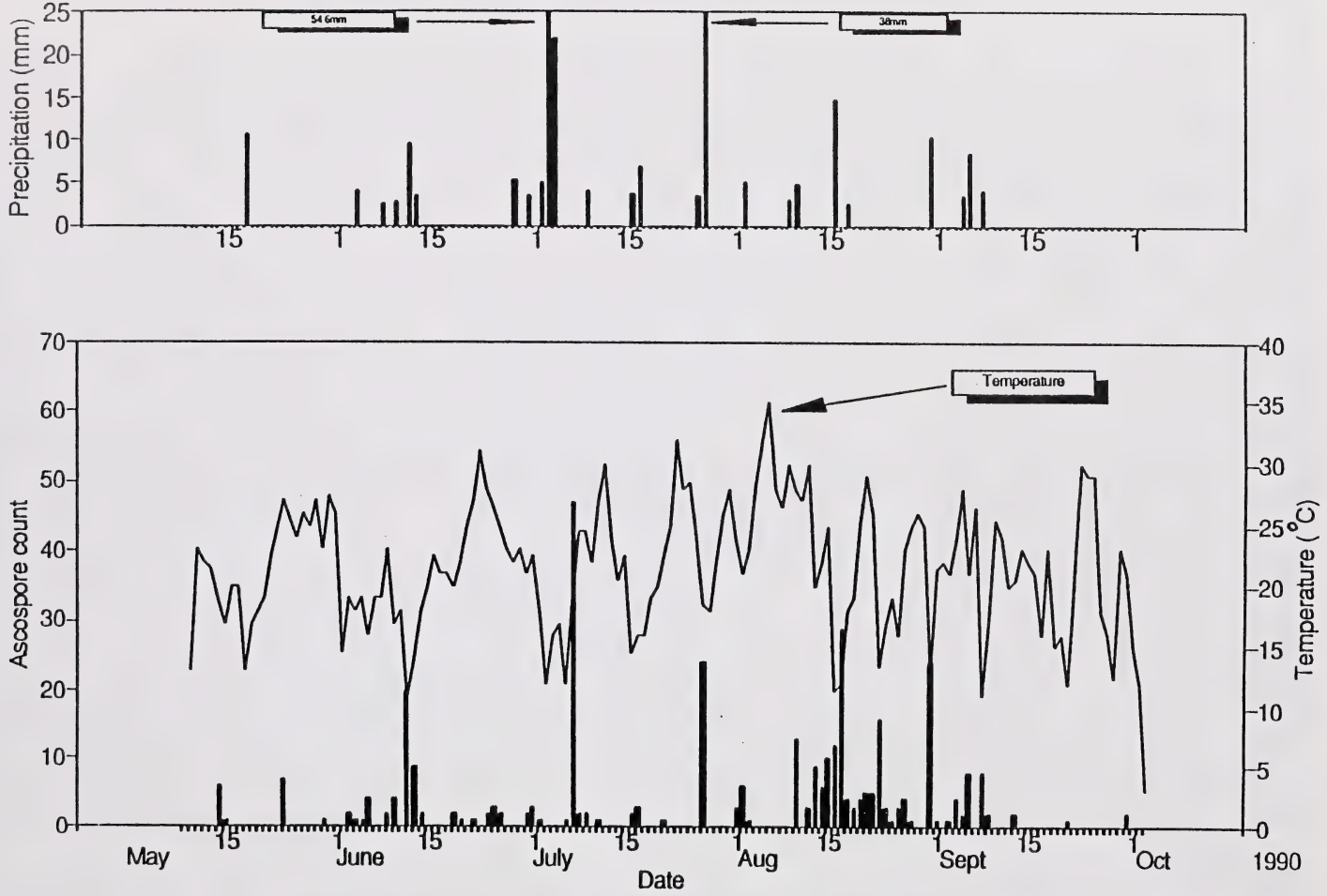


Figure 10. Daily counts of *Leptosphaeria maculans* ascospores, temperature and precipitation measured in a blackleg-infested canola field near Lloydminster, Alberta, 1990.

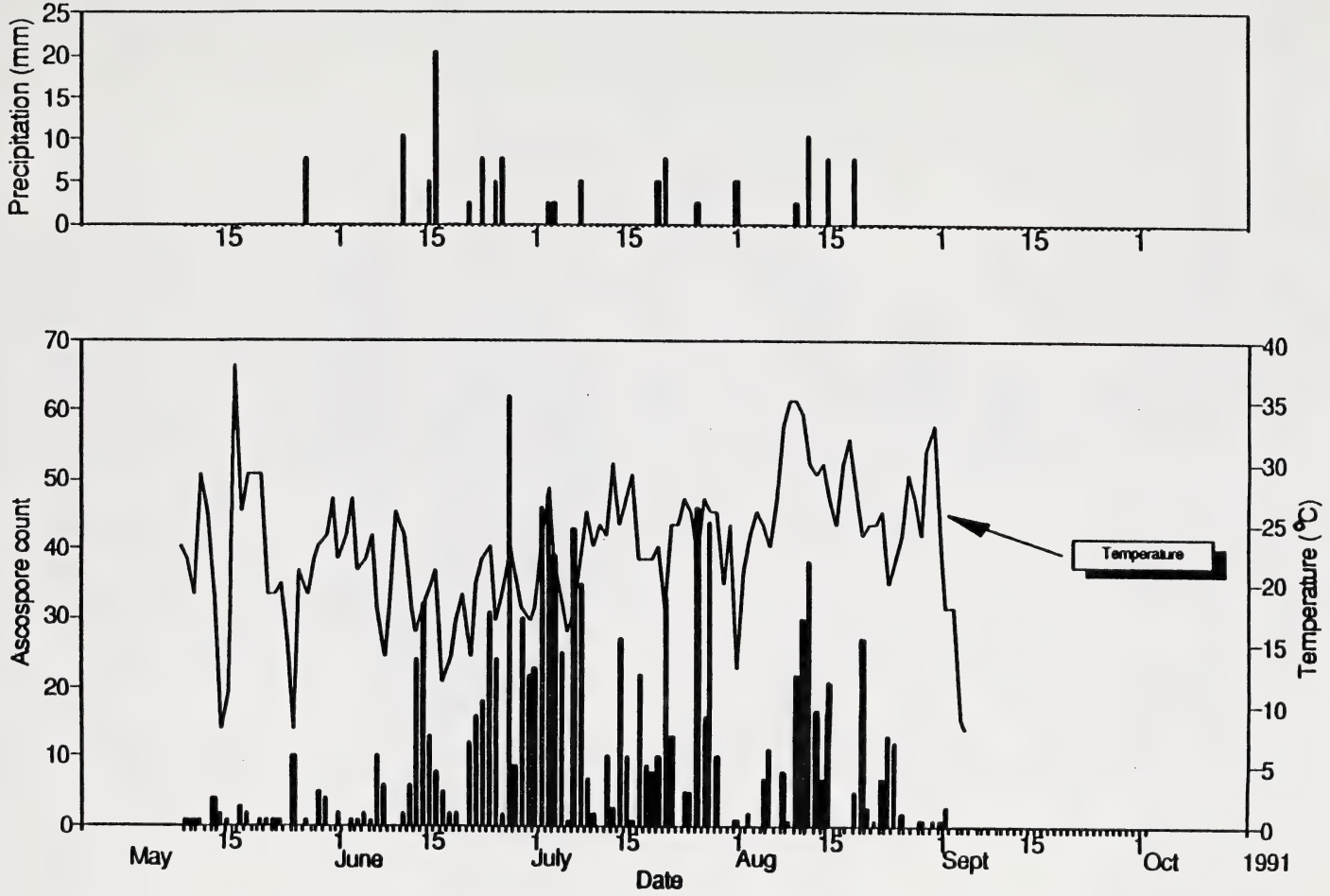


Figure 11. Daily counts of *Leptosphaeria maculans* ascospores, temperature and precipitation measured in a blackleg-infested canola field near Lloydminster, Alberta, 1991.

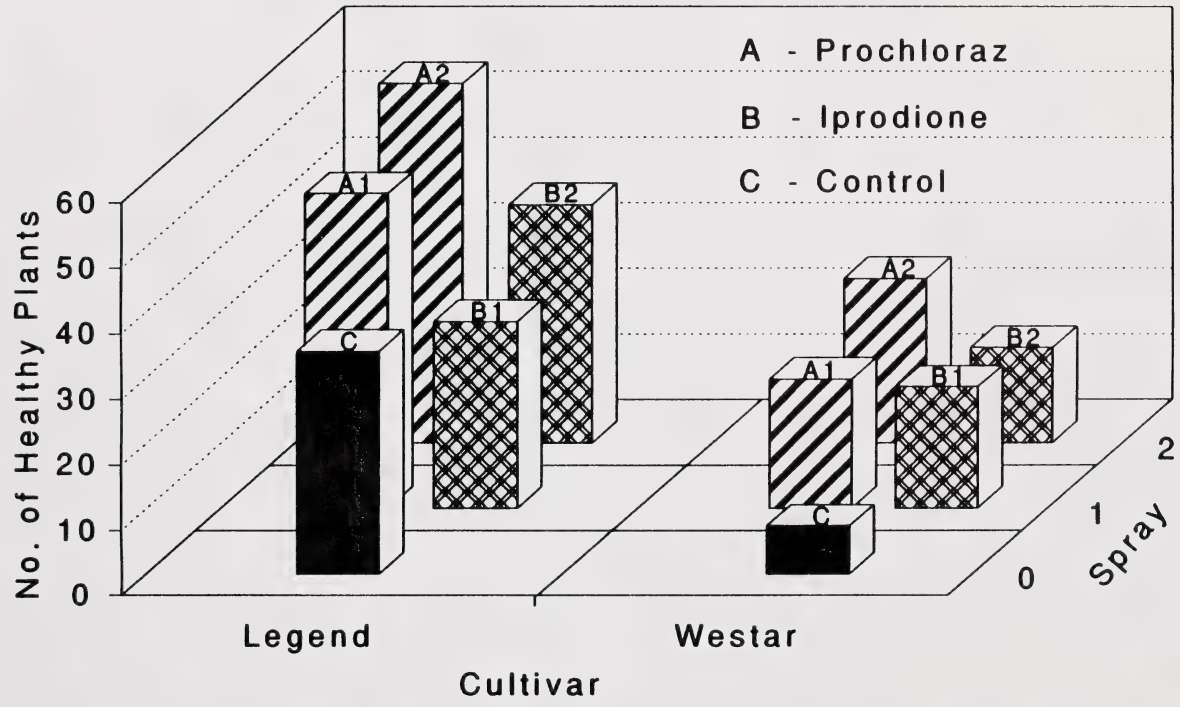


Figure 12. Number of healthy plants resulting from one or two sprays of either iprodione or prochloraz in two cultivars of canola in a blackleg-infested field, 1990.

Table 24. Effect of prochloraz (Sportak) sprays applied once at weekly intervals during the growing season on mean disease severity and mean yield per plot of *Brassica napus* cv. Legend grown in a blackleg-infested field, 1990.

Timing of spray*	Mean disease severity**	Mean yield (grams/plot)**
June 13	0.7 BC	208 A
June 20	0.8 BC	217 A
June 27	0.7 BC	223 A
July 6	0.6 C	199 A
July 13	0.9 ABC	195 A
July 20	0.8 ABC	193 A
July 27	1.1 AB	211 A
No spray	1.2 A	220 A

* Fungicide prochloraz was sprayed at 500 g ai/ha. The first date represents canola growth stage 2.4.

** Mean of four replications; means in columns followed by the same letter do not differ significantly according to Duncan's Multiple Range Test (P=0.05).

Table 25. Effect of prochloraz (Sportak) sprays applied once on different dates, at weekly intervals, during the growing season on mean disease severity and mean yield per plot of *Brassica napus* cv. Legend grown in a blackleg-infested field, 1991.

Timing of spray*	Mean disease severity**	Mean yield (grams/plot)**
June 19	2.1 A	334 A
June 26	2.3 A	314 A
July 3	2.2 A	345 A
July 10	2.2 A	286 A
July 17	2.0 A	288 A
July 24	2.3 A	305 A
July 31	2.4 A	295 A
No spray	2.4 A	324 A

* The fungicide prochloraz was sprayed at 500 g ai/ha. The first date represents canola growth stage 2.4.

** Mean of four replications; means in columns followed by the same letter do not differ significantly according to Duncan's Multiple Range Test (P=0.05).

Table 26. Mean disease severity and mean yield per plot of two cultivars of *Brassica napus*, cvs. Legend and Westar, grown in a blackleg-infested field and sprayed once or twice with iprodione (Rovral) or prochloraz (Sportak), 1990.

Cultivar	Fungicide*	# of sprays**	Mean disease severity#	Mean yield (grams/plot)#
Legend	Check	0	1.7 DE	220 AB
Legend	Iprodione	1	1.8 DE	315 A
Legend	Iprodione	2	1.7 DE	262 AB
Legend	Prochloraz	1	1.4 EF	302 A
Legend	Prochloraz	2	0.9 F	271 AB
Westar	Check	0	3.9 A	159 B
Westar	Iprodione	1	2.8 BC	192 AB
Westar	Iprodione	2	3.3 AB	189 AB
Westar	Prochloraz	1	2.6 C	214 AB
Westar	Prochloraz	2	2.2 CD	264 AB

* Each fungicide was sprayed @ 500 g ai/ha.

** First spray was applied at the growth stage 2.4; the second two weeks later.

Mean of four replications; mean in columns followed by the same letter do not differ significantly according to Duncan's Multiple Range Test (P=0.05).

Table 27. Analysis of variance of blackleg severity data in Table 26.

Source	Degree of Freedom	Sum of Squares	F-Ratio
VAR	1	21.2576	108.44**
FUN	2	6.5034	16.59**
SPRAY	1	0.1405	0.72 NS
VAR*FUN	2	1.4928	3.81 NS
VAR*SPRAY	1	0.2178	1.11 NS
FUN*SPRAY	1	0.7021	3.58 NS
VAR*FUN*SPRAY	1	0.1653	0.84 NS
Error	30	5.8807	
Total	39	36.3602	

** Significant at P = 0.01; NS = not significant.

Table 28. Mean number of healthy plants resulting from prochloraz (Sportak) or iprodione (Rovral) sprays on Legend and Westar plots in a blackleg-infested field.

Fungicide	Mean # of healthy plants*
Prochloraz	37 A
Iprodione	24 B
Check	21 B

* Means of four replications; means in columns followed by the same letter do not differ significantly according to Duncan's Multiple Range Test (P=0.01).

Table 29. Analysis of variance of number of healthy plants data shown in Figure 12.

Source	Degree of Freedom	Sum of Squares	F-ratio
VAR	1	5428.90	32.70**
FUN	2	1881.54	5.67**
SPRAY	1	124.03	0.75 NS
VAR*SPRAY	2	117.54	0.35 NS
VAR*FUN	1	344.53	2.08 NS
FUN*SPRAY	1	34.03	0.21 NS
VAR*FUN*SPRAY	1	52.53	0.32 NS
Error	30	4980.00	
Total	39	12963.10	

** Significant at P = 0.01; NS = not significant.

Several workers reported failure of fungicidal seed treatments to control blackleg of canola (McKenzie & Verma 1988, Verma & McKenzie 1982, Brown *et al.* 1976). Although Brown *et al.* (1976) observed that benomyl seed treatments (1.1% w/w) completely protected rapeseed cotyledons against *L. maculans* for 10 days after germination under greenhouse conditions, they did not investigate the protection afforded by benomyl beyond 10 days. One objective of the present study was to determine the longevity of several seed treatment fungicides in seedlings to determine if their effectiveness lasted until the crop reached the six-leaf stage and

acquired some natural resistance to blackleg (McGee & Petrie 1979). Subsequently, a foliar spray with benomyl or iprodione, registered for use against sclerotinia stem rot, might give additional control of blackleg and so integrate control of the two important diseases. Although iprodione and prochloraz were reasonably effective for reducing blackleg incidence in canola until 21 days after seeding in the growth chamber tests, in field trials all fungicides when tested 15 days after seeding were found ineffective. No information on the breakdown of these fungicides in canola is available in the scientific literature. It is likely that the concentration of these fungicides in the expanded cotyledons is too low to protect the seedling from infection by *L. maculans*. Evidently, benomyl, carbathiin or iprodione, presently registered for seed treatment on canola in Canada, do not protect seedlings from blackleg infection for a desirable period of 5-6 weeks if canola is planted on blackleg-infected stubble.

Thiabendazole and tolclofos-methyl used as seed treatments to control seed-borne *L. maculans* performed better on cv. Westar than on cv. Tobin in laboratory tests (Table 15). Significant differences between the responses of the cultivars to some of the fungicidal seed treatments is also evident from the results of ANOVA (Table 16). Perhaps the larger seeds of cv. Westar facilitate uptake of more fungicide than do the smaller seeds of cv. Tobin.

Conflicting results have been reported by several workers on control of blackleg with fungicidal foliar sprays (Barbetti 1975; Brown *et al.* 1976; Rawlinson *et al.* 1984). My results with artificially inoculated plants in field and growth chamber tests indicate that when inoculum pressure is not continuous over the growing season one spray of prochloraz + adjuvant significantly reduced the DS resulting in an increased number of healthy plants and increased yield. This fungicidal spray could be very useful to control blackleg in uninfested regions where the disease is introduced with infected seed. However, in fields where the source of primary inoculum is infested stubble, providing conidia and ascospores throughout the growing season (as confirmed by my spore-trap studies), foliar-applied fungicides may not give a satisfactory control of the disease. This is also supported by recent results of Morrall *et al.* (1988) and Rawlinson *et al.* (1984) showing poor control of blackleg with multiple applications of prochloraz in the field.

In the ANOVA for the disease severities on parts of the plant developing infection due to direct inoculation (Tables 19, 20), the highly significant F-value for the sprays revealed that over all the fungicides tested, two sprays caused a consistently greater reduction of the disease

severity relative to one spray; individually, however, each fungicide when sprayed twice did not significantly reduce the disease severity compared with that due to one spray (Table 18). The results suggest that two sprays were more effective than one spray in restricting the lesion size, but had little effect on previously established lesions. As well, the non-significant F-value for the sprays in the ANOVA results of the stem lesions, developing as a result of foliar inoculation (Table 21), indicate that the second spray had minimal effect on the fungus deep seated in the leaf tissue and systemically progressing towards the stem base (Hammond *et al.* 1985).

Canola is reported to be most susceptible to blackleg before it reaches the six-leaf stage (Gladders and Musa 1980, McGee and Petrie 1979). My results show that significant control of blackleg may not be obtained if application of an effective fungicide is delayed beyond the early stages of plant growth. Perhaps the blackleg fungus, which is present in the leaves during the early stages of infection, advances deeply enough into stem where it is not affected by the delayed application of a fungicide. As well, the known systemic fungicides do not move downwards within the plant after entering plant tissue. Therefore, there is a greater possibility that the blackleg fungus which moves down within the infected stem (Hammond *et al.* 1985) could escape action of a fungicide that is sprayed after the fungus has penetrated the stem.

Ascospores were observed as early as May 15 and as late as September 15. There was some indication in 1991 that their population increased as the growing season progressed. Canola is reported to develop some natural tolerance to the disease as the season progresses. Therefore, the influence of a larger population of ascospores in the latter half of the growing season might have been offset by the tolerance developed by the maturing crop.

The spore trapping results also suggest that the ascospore release from pseudothecia is influenced by precipitation. These results support earlier reports from Australia (McGee 1977) that rainfall of more than 1.0 mm was required for large ascospore discharges. Since canola is more susceptible to blackleg during the early stages of plant growth, precipitation during that period could appreciably increase the disease severity. Therefore, to achieve acceptable blackleg control, farmers in an infested region may have to consider spraying a fungicide when practicable within a day or so after rain showers during the first six weeks of plant growth.

Ascospore discharges have been reported to be greater in 2-year-old stubble than in 1-year-old stubble (McGee and Petrie 1979). This increased ascospore activity during the early part of growing season most likely was responsible for increased blackleg severity and poor

disease control in 1991. The findings reaffirm that crop rotation of at least 3 years, preferably 4, is required to minimize the chances of severe disease occurrence (Petrie 1986).

Blackleg of canola progresses at a faster rate at 18°C than at 12°C (Barbetti 1975). It is not known what temperature is most conducive to the ascospore discharge from pseudothecia. Most large ascospore discharges were recorded when the air temperature was about 15°C. These discharges also coincided well with rainfalls of over 5 mm. More work is required to determine the effect of temperature on survival and functioning of pseudothecia in aging blackleg-infected canola stubble. Spore trap data show that temperature more than 20°C was not very conducive to ascospore build-up.

The extent of disease reduction with a single spray of prochloraz was considerably more in Westar (from disease severity of 3.9 in check to 2.6) than in Legend (from 1.7 to 1.4) (Table 26). The results suggest that the systemic fungicides used may have limitations in fully arresting the fungal activity once the disease has been reduced to certain low levels. Also, no significant difference in disease control was found between the number of sprays; the results agree with those of growth chamber studies mentioned earlier and suggest that the second spray had little effect on the previously established lesions.

Results of two years of field studies confirm that Legend, although not totally resistant, has superior tolerance to blackleg compared to Westar which is highly susceptible. Until a suitable fungicidal control program is developed, canola growers in infested regions may have to depend upon the use of cultivars such as Legend and crop rotation of four years or more to reduce yield losses due to blackleg.

More work is needed to determine if the high fungitoxicity of some fungicides such as ethyltrianol, prochloraz or propiconazole could be used against *L. maculans* to reduce primary inoculum in an infested field by spraying the stubble.

Complete protection of young seedlings for 21 days with iprodione seed treatment (Table 17) revealed its systemicity in canola, although it is not claimed to be so by the manufacturer. It is not certain why iprodione gave good control of blackleg under growth chamber conditions but not in the field. Inactivation due to weather under field conditions, which has been reported for other fungicides (Woodcock 1977), should not be the case since iprodione is used as a foliar spray to control sclerotinia stem rot on canola.

Improved disease control was achieved when fungicides were used with an adjuvant. Adjuvants with properties of wetting agents possibly assist in the coverage of waxy plant surfaces of canola leaves resulting in increased fungicidal protection against extraneous fungi. Surfactants have also been reported to enhance penetration of stomata by pesticides (Prasad *et al.* 1967). The phytotoxic effect of ethyltrianol and propiconazole in these studies may have been due to their increased uptake by leaves with the use of adjuvants.

6.4 Biological Control

Biological control is environmentally the most prudent way of managing plant diseases. Development of new, fungicide-resistant strains of pathogens is a constant threat to any disease control program. Therefore, investigations were conducted to identify organisms that inhibit development of *L. maculans*. Of the various fungi and bacteria tested, the fungus *Penicillium verrucosum* and the bacterium *Bacillus macerans* looked very promising. Research on *B. macerans* is still in the preliminary stages, whereas we have completed the intended research on isolation, identification and biological activity of the *P. verrucosum* metabolite, citrinin, which was found to be inhibitory to *L. maculans*. The work on characterization of the metabolite was done by J.S.Dahiya, Plant Science Department, University of Alberta, Edmonton.

Citrinin is known to be a secondary metabolite of several penicillia and aspergilli. This mycotoxin, first isolated from *Penicillium citrinum* in 1931 (Hetherington and Raistrick 1931), has strong antifungal but weak antibacterial activity. It was found associated with heated grains in Saskatchewan (Scott *et al.*, 1970; Scott 1978). Its biological activity against *Sclerotinia sclerotiorum* and *Rhizoctonia solani* has been reported by Melouk and Akem (1987). In the present study we report isolation of this mycotoxin from the fungus, *P. verrucosum*; its biological activity was studied against several fungi pathogenic on canola, including *Alternaria brassicae*, *Fusarium acuminatum*, *L. maculans*, *R. solani* (AG2-1), and *S. sclerotiorum* with a view to use this *Penicillium* sp. to develop an integrated biocontrol program for canola diseases. A report on a part of this study has been published (Kharbanda and Dahiya 1990).

6.4.1 Isolation and Characterization of *Pencillium verrucosum* Metabolite

The fungus *P. verrucosum* used in the present study was recovered as an aerial contaminant inhibiting *L. maculans* (Kharbanda and Dahiya 1990). A single-conidial culture of the *Penicillium* isolate was identified as *P. verrucosum* by M.A. Klich, USDA, New Orleans, Louisiana, USA and by the International Mycological Institute, Bakeham Lane, Egham, Surrey, England.

The fungus was grown at 23 ± 2°C in potato dextrose broth on an orbital shaker set at 150 rpm for six weeks. At the end of the growth period, the fermented growth was filtered through cheesecloth, centrifuged @ 4000 rpm for 15 minutes and finally filtered through a 0.2 µm Millipore bacterial filter. The filtrate (500 mL) was dried *in vacuo*. at 30°C. The residue was redissolved in distilled water (50 mL) and partitioned against ethyl acetate (100 mL, 3x). Ethyl acetate fractions were pooled and dried *in vacuo*. The residue was re-dissolved in methanol.

The methanolic residue was purified by HPLC analysis using RP-18 (NOVA PAK column, Waters Associate) column (25.0 cm x 1.00 cm id) using a solvent $CH_3CN:H_2O$, 65:35, v/v. The LC-UV detector was set at λ 254 nm and a solvent flow rate of 2.5 mL/min. Samples corresponding to peaks on the chart recorder were collected, dried, and subjected to spectral analysis.

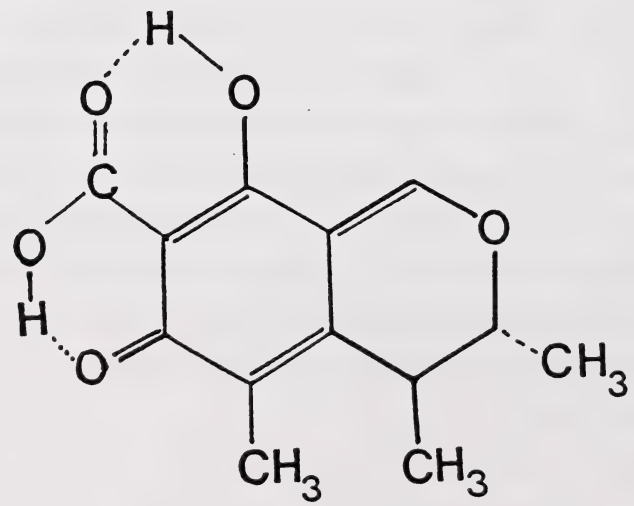


Figure 13. Chemical structure of citrinin.

6.4.2 Spectral Analysis

UV spectrum in absolute methanol was recorded on Varian XL-200 spectrophotometer. ^{1}HNMR ($CDCl_3$400 MHz) was recorded on Brukar Model WH-90 using TMS as an internal standard. The mass spectrum (70 eV) was recorded on VG-analytical 11-250J by direct probe insertion. IR spectrum was recorded on Beckman Spectrometer using nujol.

6.4.3 Biological Activity of the Metabolite

The inhibitory effect *P. verrucosum* was tested against *A. brassicae, F. acuminatum, S. sclerotiorum, L. maculans* (virulent strain), *L. maculans* (weakly-virulent strain), and *R. solani* (AG2-1). These fungi were originally isolated from infected canola plants. Two experiments were conducted: In the first, 100 mL petri plates containing 20 mL of potato dextrose agar (PDA) were flooded with about 3 mL of a macerated culture suspension containing conidia and/or mycelial fragments of the test fungus. The plates were gently swirled to cover the agar surface evenly, and the excess suspension was decanted; two hours later, a 4-mm-diameter mycelial, PDA-plug from an actively growing culture of *P. verrucosum* was placed at the centre of each plate. Four replications for each fungus tested were arranged in randomized complete block design and incubated at 23°C in the dark for seven days. The fungi tested were then observed for zone of inhibition around the *P. verrucosum* colony in each plate.

In the second experiment, the *P. verrucosum* metabolite purified and concentrated in methanol was dried under vacuum and resuspended in sterile distilled water resulting in a concentration of 2mg/mL. One mL aliquots of this concentrated suspension were dispensed into 20 mL petri plates and mixed thoroughly with 19 mL of molten PDA at about 45°C. Sterile distilled water without chemical or a suspension containing pure citrinin (Aldrich Chemical Co., Milwaukee, Wisconsin, USA) in sterile distilled water were used as control. On the solidified agar, a 4 mm mycelial PDA-plug from an actively growing culture of a test fungus was placed at the centre of each plate. Colony diameters were measured 21 days after incubation at 21°C in the dark.

6.4.3.1 Effect of Heat on Metabolite

Five mL aliquots of the purified metabolite (conc. 1 mg/mL) were autoclaved at 121°C for 20 minutes. The biological activity of the autoclaved metabolite was tested against *R. solani* and *L. maculans* following the procedure described for the experiment above.

6.4.3.2 Inhibitory Effect on Blackleg of Canola

A conidial suspension of *P. verrucosum* (2 x 10^{6-7} conidial /mL) was mixed with an equal amount of a suspension of pycnidiospores of *L. maculans* (2 x 10^6 pycnidiospores/mL) so as to obtain a final concentration of 50 mg/mL of the metabolite in sterile distilled water. The second fully expanded leaves of canola plants cv. Westar (*B. napus*) were inoculated at four isolated spots by puncturing the leaf with a hypodermic needle and depositing either a mixture of conidiospores + pycnidiospores, pycnidiospores alone, conidiospores alone, or sterile distilled water; four leaves on separate plants were inoculated for each treatment. The inoculated leaves were individually covered with plastic bags to maintain high humidity, and incubated at 21°C for 48 hours in a growth chamber. Final data on disease development were recorded 21 days after incubation.

6.4.3.3 Biological Control: Results and Discussion

The antifungal substance was obtained as yellow crystals from aqueous methanol fraction of crude extract. On mass spectral analysis the purified compound gave a molecular ion M^+ 250 (89.0) $C_{13}H_{14}O_5$, 250.0342) with other fragments (M^+ -18,H_2O) 217(65) (M^+ -CH), 206(100), 191(22.8), 175(19), 161(9), 146(7), 133(5.8), 103(3.9), 91(32), 77(28), 65(18), and 51(22.5) (Figure 14,15). UV spectral analysis in absolute methanol gave λ_{max}^{MeOH} (nm), 251, and 331. ^1H NMR spectral analysis in $CDCl_3$ gave a doublet at ς 1.26 and ς 2.08. A quintet at ς 4.10 and triplet at 4.82 were due to methyl (-CH_3) protons. A singlet at ς 15.89 confirms the -COOH proton. Presence of >C=0 was further confirmed by IR spectral analysis data of 1610 cm^{-1}. Presence of aromatic protons was given by singlets at ς 7.26 and ς 7.42. Based on UV, IR NMR, and mass spectral data the compound was identified as citrinin (Figure 13) already described from *Aspergillus terreus* and *P. citrinum* (Hetherington and Raistrick 1931).

This is the first report on citrinin production by *P. verrucosum* in North America. Citrinin has been associated with mycotoxicoses in farm animals (Scott *et al.*, 1970; Scott 1978). Under natural field conditions, as happens with *Fusarium* spp., there may be certain factors that trigger production of mycotoxin by *P. verrucosum*. Therefore, the animal feed contaminated with this fungus could be potentially hazardous to livestock and unfit for consumption.

The metabolite was heat stable and autoclaving at 121°C for 15 minutes did not destroy its inhibitory effect on growth of *Rhizoctonia solani* (Table 30). It exhibited strong antifungal activity against *L. maculans* in petri plate tests. The mean colony diameters of virulent and weakly virulent isolates of *L. maculans* on PDA mixed with metabolite were 35 mm and 45 mm, respectively, compared with 42 mm and 52 mm on the unamended substrate. As well, the severity of blackleg lesions on leaves was significantly less when canola leaves were inoculated with a mixture of conidia of both *L. maculans* and *P. verrucosum* compared with the disease severity caused by *L. maculans* alone (Table 31, Plate 12). Evidently, *P. verrucosum* effectively controlled blackleg. Citrinin has also been reported to inhibit *R. solani*, and certain strains of *Sclerotinia* (Melouk and Akem 1987, Kharbanda and Dahiya 1990). However, the reported nephrotoxic nature of citrinin (Scott *et al.*, 1970) makes it a non-candidate for biocontrol of blackleg and other diseases of canola.

Table 30. Influence of heat on the biological activity *Penicillium verrucosum* metabolite against *Rhizoctonia solani*.

Treatment	Mean colony diameter (mm)*
Autoclaved (121°C, 15 mins.)	61 b
Non-autoclaved	62 b
No metabolite	76 a

* Column mean followed by the same letter are statistically not different according to Duncan's Multiple Range Test (P=0.05).

Table 31. Severity of blackleg on canola leaves inoculated with *Leptosphaeria maculans* conidia alone or in a mixture with conidia of *Penicillium verrucosum*.

Treatment	Mean blackleg severity*
Leptosphaeria maculans alone	2.7 a
Leptosphaeria maculans + *Pencillium verrucosum*	2.1 b
Pencillium verrucosum alone	0 c
check (water only)	0 c

* Blackleg severity scale: 0 = pycnidia absent; 1 = pycnidia present, lesion < 5 mm; 2 = pycnidia present, lesion ≥ 5 mm < 10 mm; 3 = pycnidia present, lesion > 10 mm. Column means followed by the same letter are not significantly different according to Duncan's Multiple Range Test (P=0.05).

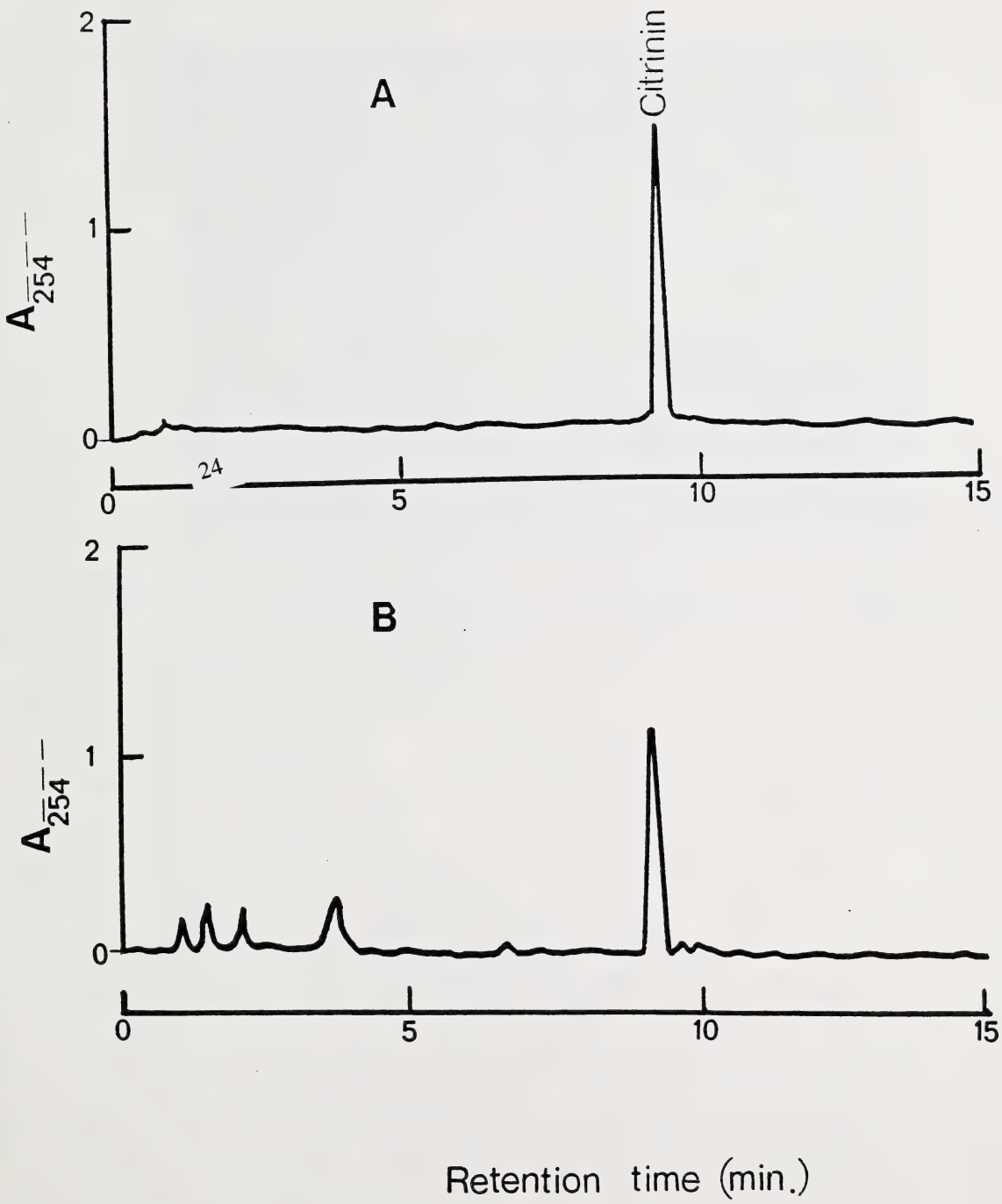


Figure 14. HPLC trace of *Penicillium verrucosum* metabolite (B) and of citrinin (A).

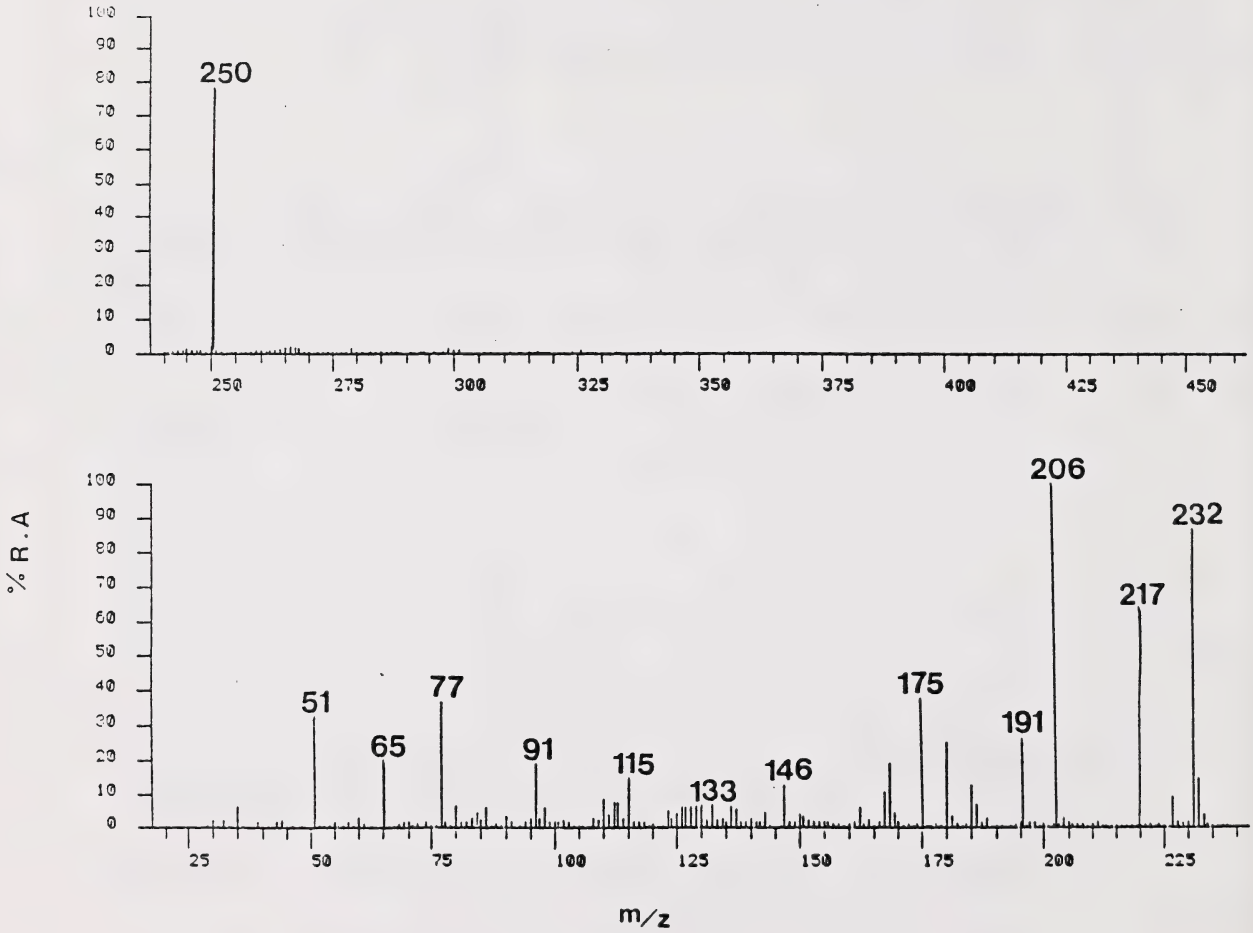


Figure 15. Mass spectrum of *Penicillium verrucosum* culture filtrate (M^+ 250).

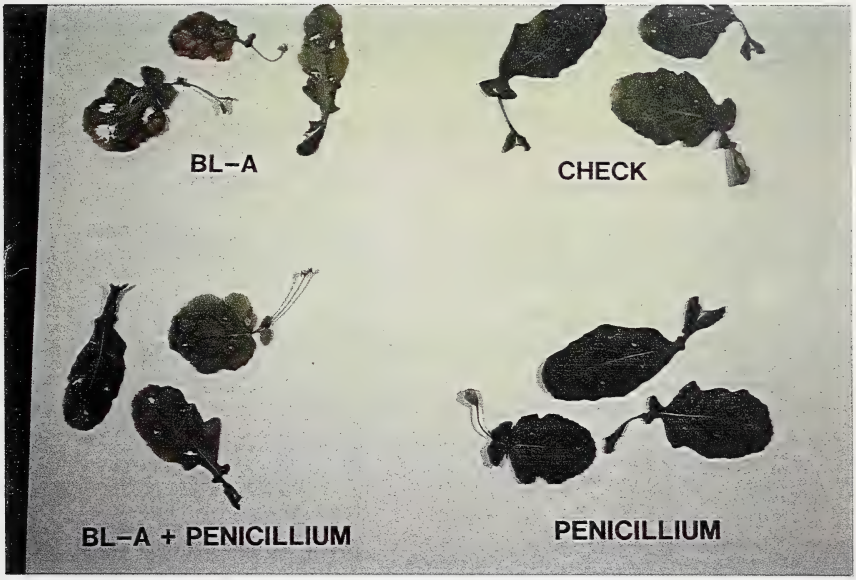


Plate 12. Blackleg lesions on canola leaves inoculated with *Leptosphaeria maculans* (isolate BL-A) conidia alone or in a mixture with conidia of *Penicillium verrucosum*.

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